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PATENT
Customer No. 22,852
Attorney Docket No. 3495.0068-10

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:)	
Luc MONTAGNIER et al.)	Group Art Unit: 1648
Application No.: 09/739,289)	Examiner: Jeffrey S. Parkin
Filed: December 19, 2000)	
For: IMMUNOLOGICAL REAGENTS)	Confirmation No.: 3312
AND DIAGNOSTIC METHODS)	
FOR THE DETECTION OF)	
HUMAN IMMUNODEFICIENCY)	
VIRUS TYPE 2 UTILIZING)	
MULTIMERIC FORMS OF THE)	
ENVELOPE PROTEINS GP300,)	
P200, AND P90/80)	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION OF JACQUES H. M. COHEN, M.D., PH.D., UNDER 37 C.F.R. § 1.132

I, Jacques H. M. Cohen, M.D., Ph.D., do hereby make the following declaration:

1. I do not have any ownership interest or interest as an inventor in this application.
2. My Curriculum Vitae, and a list of publications that I have authored or coauthored, is attached hereto as Exhibit 1.
3. I hold State Doctorate in Medicine and Ph.D. in Human Biology degrees from the University Claude Bernard of Lyon. I held the position of Assistant Professor of the Universities - Hospital Practitioner, at Reims, from October 1986 to July 1990.

Since 1990 I have held the position of Professor of the Universities - Hospital Practitioner, since 1993 the position of Section Head of the Laboratory of Immunology, CHU Reims, and since 2000 the position of Section Head of the Bacteriology, Virology, and Hygiene Laboratories. My research focus has included histocompatibility and autoimmune pathology, dealing with HIV infected patients and anti-HIV and hepatitis humoral immunity. I participated in the discovery of the O-subtype of HIV-1.

4. On information and belief, attached hereto as Exhibit 2 is a copy of U.S. patent application Serial No. 09/739,289 ("the '289 application").

5. I have read and understood the contents of the attached copy of the '289 application.

6. On information and belief, the '289 application was filed on December 19, 2000.

7. On information and belief, the '289 application is a continuation of U.S. patent application Serial No. 08/321,566, filed October 27, 1994.

8. I have been asked to consider whether the '289 application describes an antibody that binds specifically to one or more HIV-2 proteins selected from gp300, p200, and p90/80, but that does not bind to the HIV-2 protein gp140.

9. Based on my understanding of the specification of the '289 application and my experience in the field of immunology, the '289 application describes an antibody that binds specifically to one or more HIV-2 proteins selected from gp300, p200, and p90/80, but that does not bind to the HIV-2 protein gp140.

10. My understanding is based, in part, on passages found on pages 4, 5, and 58 of the '289 application.

11. At page 4, line 21 to page 5, line 9, the '289 application states:

Four glycoproteins of apparent molecular weights 300,000, 140,000, 125,000, and 36,000 daltons (gp300, gp140, gp125, and gp36) are detectable in human immunodeficiency virus type 2 (HIV-2) infected cells. The gp125 and gp36 are the external and transmembrane components, respectively, of the envelope glycoproteins of HIV-2 mature virions. It has now been discovered that the gp300 is a dimeric form of gp140, which is the precursor of HIV-2 envelope glycoprotein. This invention thus provides gp300 glycoprotein of HIV-2 and human retroviral variants of HIV-2 in purified form.

This invention also provides proteins of HIV-2 or of a human retroviral variant of HIV-2 having apparent molecular weights of about 200 Kd (p200) and about 90 to about 80 Kd (p90/80). These proteins are substantially unglycosylated and are in a purified form.

12. I understand this passage as describing newly identified, purified gp300, p200, and p90/80 proteins of HIV-2 as a subject of the invention described in the '289 application. By making reference to the gp140 protein of HIV-2, but not referring to it as part of the invention, this passage also conveys that the gp140 protein is not a subject of the invention described in the '289 application.

13. At page 58, lines 11-13, original claim 3 of the '289 application states:

3. An isolated antibody which binds with a protein selected from the group consisting of gp300 of HIV-2, p200 of HIV-2, p90/80 of HIV-2, and gp300_{SV}.

14. I understand this passage as describing antibodies, which bind with the newly identified, purified gp300, p200, and p90/80 proteins of HIV-2 as a further subject

of the invention described in the '289 application. Because the '289 application makes reference to the gp140 protein of HIV-2, but does not refer to it as part of the invention, and because the '289 application specifically states that the antibodies described in the application bind with a protein selected from gp300 of HIV-2, p200 of HIV-2, and p90/80 of HIV-2, without mentioning binding with gp140 of HIV-2, I understand this passage as describing antibodies that bind with gp300 of HIV-2, p200 of HIV-2, and/or p90/80 of HIV-2, but not with gp140 of HIV-2.

15. My understanding of the meaning conveyed by the passages of the '289 application described above is consistent with what was known in the field of immunology before October 27, 1994. For example, Mollnes et. al., "Monoclonal Antibodies Recognizing a Neoantigen of Poly(C9) Detect the Human Terminal Complement Complex in Tissue and Plasma," *Scand. J. Immunol.*, Vol. 22, pp. 183-195 (1985), a copy of which is attached hereto as Exhibit 3, described antibodies that bind to the terminal complement complex (TCC), but not to the individual components of the complex. (Exhibit 3 at Abstract.) The TCC consists of components C5b, C6, C7, C8, and C9. (Exhibit 3 at page 183, col. 1.) As described at page 183 of Exhibit 3, neoantigens are new antigenic determinants that appear in the TCC upon its assembly, whereas they cannot be detected in the individual native components. Prior to Mollnes et al., other workers had described other antibodies and antisera that recognize neoantigens present in the TCC but absent from the individual components when not complexed to form the TCC. (Exhibit 3 at page 183, col. 2.) As described at page 190 of Mollnes et al., two monoclonal antibodies that the authors investigated in detail were

found to react much more strongly with polymerized C9, which is used as a proxy for the TCC complex, than with monomeric C9. (Exhibit 3 at page 190, col. 2.)

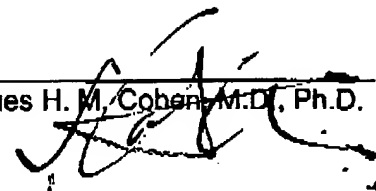
16. Similar results were reported by other authors. For example, Hugo et al., "Monoclonal Antibodies Against Neoantigens of the Terminal C5b-9 Complex of Human Complement," *Bioscience Reports*, Vol. 5, pp. 649-658 (1985), a copy of which is attached hereto as Exhibit 4, describes analysis of four monoclonal antibodies against C9-dependent neoantigens of human C5b-9 (a soluble form of the TCC). (Exhibit 4 at Abstract.) As described in the article, the antibodies are specific to neoantigens found in the TCC and do not bind to the individual components of the TCC. (Exhibit 4.) As another example, Falk et al., "Neoantigen of the Polymerized Ninth Component of Complement," *J. Clin. Invest.*, Vol. 72, pp. 560-573 (1983), a copy of which is attached hereto as Exhibit 5, describes a monoclonal antibody that binds to a neoantigen present on the C9 portion of the TCC (referred to in article as "MAC"). The antibody binds to an antigen formed after the C9 component of the TCC binds with the intermediate C5b-8 complex, but does not bind with the individual C5, C8, and C9 components or with the intermediate C5b6 and C5b67 complexes. (Exhibit 5 at page 564, col. 2.)

17. The studies described in Exhibits 3-5 demonstrated prior to 1994 the principle that neoantigens, present in molecular complexes but absent from the components of the complexes when in free form, can be recognized by specific antibodies. Antibodies to the neoantigen are necessarily able to bind to the protein complex that contains the neoantigen but not to the components of the complex when in free form. For this reason, upon reading the '289 application I understand today and

would have understood in 1994 that the antibodies of the invention are antibodies that bind with gp300 of HIV-2, p200 of HIV-2, and/or p90/80 of HIV-2, but not with gp140 of HIV-2. Because gp300 is a dimeric form of gp140, I understand today and would have understood in 1994 that these antibodies distinguish between the dimeric gp300 complex and the gp140 monomer by binding to a neoantigen present on the gp300 complex but absent from the gp140 monomer.

18. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 4/17/05

By: 
Jacques H. M. Cohen M.D., Ph.D.

CURRICULUM VITAE

NAME and first name: COHEN Jacques H.M.
Date and birth place: March 18th, 1951 in Paris
University Professor and Hospital Practitioner
Section Head of the central laboratory of Immunology, CHU (University Hospital Complex) of Reims (France)
Interim section Head of the Bacteriology - Virology - Hygiene laboratories, at the CHU of Reims

GRADUATE STUDIES:

University of Reims - Faculty of medicine (1968-1969)
University Claude Bernard, Lyon - Faculty of medicine A. Carrel - (1969-1990)

UNIVERSITY DIPLOMAS:

State Doctorate in Medicine - Lyon September 22nd (1983) "Sero-epidemiological study of anti-Epstein-Barr antibodies and of the HLA antigens in rheumatoid poly-arthritis disease" Thesis of Medicine - University Claude Bernard - Lyon No 316 (1983). Highest level of distinction for a doctorate.
CES in Rheumatology (by equivalent qualification) (1982).
COMPETENCE in Nephrology - National Committee of the French medical association board (1991)
CES in Immunology of clinical biology (1977)
Certificate in Immuno-hematology and Immuno-pathology (Pasteur Institute) (1978)
CES in Hematology of clinical biology (1979)
CES in General Immunology of clinical biology - University Claude Bernard, Lyon (1980)
Master in Human Biology Immunology - University Claude Bernard, Lyon (1980)
AEA in Immunology. Mention "Applied immunology to pathologic processes" University Claude Bernard, Lyon
DEBII in Immunology. Mention "Applied immunology to pathologic processes" University Claude Bernard, Lyon
(1983). Contribution to the sero-epidemiological study of the antigen RANA (Rheumatoid arthritis nuclear antigen)
Diploma of Study and Research in Human Biology
Ph.D. in Human Biology from the University Claude Bernard of Lyon I. "Contribution to the study of the receptors CRI (CD35) and CR2 (CD21) of the fragments C3b and C3d of the fraction C3 of the complement" (1989)
H.D.R (Accreditation to supervise research activities).

SUCCESSIVE FUNCTIONS:

INTERN CHU REIMS, with a one-year training period in the CHU of Lyon.
RESEARCH ASSISTANT INSERM in a host position for Intern in Medicine INSERM U80. Director Pr TRAEGER, team of Pr REVILLARD (1983 - 1983).
HOSPITAL ASSISTANT - UNIVERSITY ASSISTANT IN IMMUNOLOGY - Lyon. With Pr Traeger for the university part and with Pr Révillard for the hospital part (1983 - 1984).
HOSPITAL ASSISTANT AND UNIVERSITY ASSISTANT IN IMMUNOLOGY - Reims. With Pr DROPSY (December 1984 - October 1986).
ASSISTANT PROFESSOR OF THE UNIVERSITIES - HOSPITAL PRACTITIONER - Reims. From October 1st, 1986 (entrance exam in 1984) to July 1990.
PROFESSOR OF THE UNIVERSITIES - HOSPITAL PRACTITIONER - Reims, (1990)
SECTION HEAD of the Laboratory of Immunology, CHU Reims (1993), and interim SECTION HEAD of the Bacteriology - Virology - Hygiene laboratories - CHU Reims (2000)
SCIENTIFIC MISSION for the French Ministry of National Education, Research and Technology (1999)
MEMBER OF THE SCIENTIFIC BOARD OF THE SANITARY SECURITY AGENCY FOR THE HEALTH PRODUCTS (AFSSAPS) (1999).
IN CHARGE OF THE THEME "GENETIC THERAPY" OF THE FRENCH-ISRAELI COOPERATION within the scope of the AFIRST (French Israeli Association for Scientific and Technique Research) (1999)
LEGAL EXPERT, Court of Appeal of Reims (2000)
LEGAL EXPERT, Administrative Court of Appeal of Paris (July 7th 2004)

20,000 2000 11:00 AM

ACTIVITY: During my **Medicine Studies Internship**, I have specialized in **Rheumatology** and **Nephrology**, both disciplines being related to Immunology to which I have chosen to turn to while still having, until today, duties in intensive care in Nephrology and Transplantation (one week-end per month), as well as being a consultant in clinical Immunology.

My research activities were oriented towards to **normal immune and antiviral pathological responses** related to the Epstein-Barr virus, which were the subject of my Thesis in Medicine, my DERBH (Diploma of Study and Research in Human Biology) and my Thesis in Human Biology.

From 1986 to 1990, in a hospital environment, I have assured the practical responsibilities of a **Virology-Immunology laboratory** and I have developed an interest for the Immunology of Hepatitis and HIV, which allow me to participate in the discovery of the O-subtype of the HIV-1. Since 1991, I am in charge of an **Immunology laboratory** working on subjects covering histocompatibility and auto-immune pathology, dealing with HIV infected patients and anti-HIV and hepatitis humoral immunity. I am an active member of the Auto-Immunity Study Group (**GEAI**), the Board of Directors of France-Transplant, France-Greffe de Moëlle and Est-Transplant (**EFG**).

My research activities in the area of the **Complement receptors** have dealt with the function of the CR1 receptors in the immune Complex related pathologies, the **recombinant protein engineering**, particularly a multi scFv antiRh(D) and a CD4 multimer, and with the **HIV variants** and abnormal immune responses towards this virus. I have also been interested in the **HLA and diseases** associations in the Birdshot type Chorioretinopathy model.

Amongst **64 publications** published in international reviews with reading committee, I am first co-author and then last co-author in two third of these publication. I am a co-inventor of the **French HIV-O patents**.

I am a member of the Expert Groups of Drugs agency for the sections : "blood derived stable products", then "Biology / Biotechnology" and "Vaccines".

I am also active in the humanitarian area as the person in charge of the Biology Group of **Médecins du Monde** and as the designer of the "**Ultra Light Mobile Laboratory**"

(Laboratoire Mobile Ultra Léger) essentially used for infectious emergencies in the Developing Countries.

I have been working as an **official representative** of the French Ministry of National Education, Research and Technology at the DT/A2, from December 1998 to December 2002.

I have been appointed to the **Conseil Scientifique** (Scientific Board) of the Agence Française des Produits de Santé (French Agency for Health Products) in June 1999 and December 2003.

In 2003, I was in Hungary on a study assignment with the task of setting up the Franco-Hungarian cooperation in biotechnology.

I am in charge of the biology group of "Médecins du Monde". As an official representative of the French Ministry of Research in the Bio-Engineering area, I have contributed to set up the program "apport des satellites en santé et action humanitaire" and as the General Secretary of the GIS LEDA (Group of Scientific Interest, Link Education Diagnostic Assistance), I lead the setting up of the telemedicine LEDA system for the Developing Countries. This system hinges on an Internet site based at the Institut Pasteur in Paris and field interventions, for instance to set up a long-distance assisted screening for uterine cervix cancer in Cambodia.

I am presently the Director of the EA 3309 "Equipe Physiopathologie Dysimmunitaire Humaine" at the IFR 53 of the Pôle Biomolécules at the University of Reims-Champagne-Ardenne.

Reims, March 25, 2004

Pr. Jacques COHEN

PUBLICATIONS LIST

Professor Jacques H.M. COHEN

PUBLICATIONS IN INTERNATIONAL JOURNALS:

-PRI-1- J.H.M. COHEN, E. BOUVERET, M. PLUOT, and J. P. REVILLARD. Characterization of Langerhans cells using a monoclonal anti HLA DR (Ia-like) antibody. Arch. Pathol. Lab. Med. 105, 438 (1981).

-PRI-2- J.H.M. COHEN, L. JOYCE, F. BLANCHARD, J.C. ETIENNE, J. GOUGEON and G.E. DROPSY. Detection and measurement of Rheumatoid factor using a new immunoenzymatic technique with peroxidase/antiperoxidase complex. Ann. Immunol. 132 C, 201-210 (1981).

-PRI-3- D. SCHMITT, J. BROCHIER, J.H. COHEN and J.P. REVILLARD. Langerhans' cells and identification with antibodies. Arch. Pathol. Lab. Med. 106, 255-256 (1982).

-PRI-4- J.H.M. COHEN, G.M. LENOIR, Epstein-Barr virus and Rheumatoid arthritis : are RANA anti EBNA different ? Biomedecine 36, 246-249 (1982).

-PRI-5- J.P. MELIN, P. LEMAIRE, P. BIREMBAUT, L. AUBERT, J.H. COHEN, B. LARDENNOIS, and J. CHANARD. Polyarteritis nodosa with bilateral ureteric involvement. Nephron 32, 87-89 (1982).

-PRI-6- F. BLANCHARD, S. BRIANCON, J.H. COHEN, G. BETHEVENOT and F. REYES. Prognostic factors in angioimmunoblastic lymphadenopathy. Lancet i, 1449-1450 (1983).

-PRI-7- J.H.M. COHEN, P. BOUIC, D. SCHMITT, G. LENOIR and J.P. REVILLARD. Endocytosis of class II histo-compatibility antigen and formation of intra-cytoplasmic granules at the final differentiation stage of human B lymphocytes. Immunology Letters, 7, 123-127 (1983).

-PRI-8- J.H.M. COHEN, L. DANIEL, G. CORDIER, S. SAEZ and J.P. REVILLARD. Sex steroid receptors in peripheral T cells : Absence of androgen receptors and restriction of estrogen receptors to OKT 8-positive cells. J. Immunol., 131, 2767-2771 (1983).

-PRI-9- J.H.M. COHEN, T.L. VISCHER, J. CARQUIN, F. BLANCHARD, V. VON FLIEDNER, and M. JEANNET. HLA-DR antigens and the antibody response against Epstein-Barr virus. Tissue Antigens, 23, 156-162 (1984).

-PRI-10- J.H.M. COHEN, C. VINCENT, J.P. MAGAUD, G.M. LENOIR and J.P. REVILLARD. Immunoglobulin secretion by Burkitt's lymphoma cell lines. Arch. Pathol. Lab. Med. 108, 528 (1984).

-PRI-11- J.H.M. COHEN, L. DANIEL and J.P. REVILLARD. Natural killer cells and estrogen receptors. *J. Immunol.*, 132, 3229-3230 (1984).

-PRI-12- J.H.M. COHEN, T.L. VISCHER, J. CARQUIN and F. BLANCHARD. A subset of rheumatoid arthritis patients have a pattern of EPSTEIN-BARR virus antibodies like in immunodeficiency. *Arthritis Rheum.* 28, 339-340 (1985).

-PRI-13- M. REYNES, J.P. AUBERT, J.H.M. COHEN, J. AUDOIN, V. TRICOTTEY, J. DIEBOLT and M.D. KAZATCHKINE. Human Follicular Dendritic cells express CR1 CR2 and CR3 complement receptor antigens. *J. Immunol.*, 135, 2687-2694 (1985).

-PRI-14- L. DANIEL, M. MENOUNI, J.H.M. COHEN, J.P. MAGAUD, G.M. LENOIR, J.P. REVILLARD and S. SAEZ. Distribution of Androgen and Estrogen receptors among lymphoid and haemopoietic cell lines. *Leukemia Research*, 9, 1377-1378 (1985).

-PRI-15- J.H.M. COHEN, E. FISCHER, M.D. KAZATCHKINE, J. BROCHIER and J.P. REVILLARD. Characterization of monoclonal anti human-B-cell antibody BL -13 as an anti-c3d receptor (CR2) antibody. *Scand. J. Immunol.*, 23, 279-285, (1986).

-PRI-16- J.H.M. COHEN, G.E. DROPSY. Blot strips for easy LAV scale immuno-blotting. *J. Immunol. Methods*, 92, 285, (1986).

-PRI-17- J.H.M. COHEN, J.P. REVILLARD, J. MAGAUD, G.M. LENOIR, M. VUILLAUME, A. MANEL, C. VINCENT and P. BRYON. B Cell maturation stages of BURKITT'S lymphoma cell lines according to Epstein-Barr virus status and type of chromosome translocation. *JNCI*, 78, 2, 235-242, (1987).

-PRI-18- J.H.M. COHEN, J.P. AUBRY, M.H. JOUVIN, J. WIJDENES, J. BANCHEREAU, M. KAZATCHKINE and J.P. REVILLARD. Enumeration of CRI complement receptors on erythrocytes using a new method for detecting low density cell surface antigens by flow cytometry. *J. Immunol. Methods*, 99, 53-58, (1987).

-PRI-19- J.H.M. COHEN, E. FISCHER, M.D. KAZATCHKINE, G.M. LENOIR, C. LEFEVRE-DELVIN COURT and J.P. REVILLARD. Expression of CRI and CR2 complement receptors following Epstein-Barr Virus (EBV) infection of Burkitt's lymphoma cell lines. *Scand. J. Immunol.* 25, 587-598, (1987).

-PRI-20- J.H.M. COHEN, Y. LAMBREY, and G.E. DROPSY. Instantaneous roll-blot from cellulose acetate after electrophoresis. A versatile tool for monoclonal antibody characterization. *J. Immunol. Methods*, 104, 25-30, (1987).

-PRI-21- J.H.M. COHEN, J.P. AUBRY, J. BANCHEREAU, J.P. REVILLARD. Identification of cell subpopulations by dual color surface immunofluorescence using biotinylated and unlabeled monoclonal antibodies. *Cytometry*, 9, 303-309, (1988).

-PRI-22- C. FESSARD, O. RICHE, and J.H.M. COHEN. Intramuscular versus subcutaneous injection for hepatitis B vaccine. *Vaccine*, 6, 469, (1988).

-PRI-23- J.C. BRIOLAY, J.L. RODDE, J.H.M. COHEN, M. GIOUD, B. ROUX, B. KALIS and J.C. MONIER. Antinuclear antibodies detected by indirect immunofluorescence on HEp2 cells and by immunoblotting in patients with systemic sclerosis. *Journal of Autoimmunity*, 2, 165-176, (1989).

-PRI-24- J.H.M. COHEN, V. CAUDWELL, M. LEVI-STRAUSS, P. BOURGEOIS, and M.D. KAZATCHKINE. Genetic analysis of CRI expression on erythrocytes of patients with systemic Lupus Erythematosus. *Arthritis Rheum.*, 32, 393-397, (1989).

-PRI-25- J.H.M. COHEN, J.P. AUBRY, J.P. REVILLARD, J. BANCHEREAU and M.D. KAZATCHKINE. Human T lymphocytes expressing the C3b/C4b complement receptor type one (CRI, CD35) exclusively belong to FC gamma receptor-positive CD4 positive T cells. *Cellular Immunology*, 121, 383-390, (1989).

-PRI-26- J.H.M. COHEN, C. GEFFRIAUD, V. CAUDWELL and M.D. KAZATCHKINE. Genetic analysis of CRI (the C3b complement receptor, CD35) expression on erythrocytes of HIV-infected individuals. *AIDS*, 3, 397-399, (1989).

-PRI-27- T. TABARY, P. LE HOANG, H. BETUEL, N. OZDEMIR; R. SEMIGLIA, C. EDELSON and J.H.M. COHEN. Susceptibility to Birdshot chorioretinopathy is restricted to the HLA A-29.2 subtype. *Tissue Antigens*, 36, 177-179, (1990).

-PRI-28- P. CORNILLET, F. PHILBERT, M.D. KAZATCHKINE and J.H.M. COHEN. Genomic determination of the CR1 (CD35) density polymorphism on erythrocytes using polymerase chain reaction amplification and Hind III restriction enzyme digestion. *J. Immunol. Methods*, 136, 193-197, (1991).

-PRI-29- T. TABARY, A. PROCHNICKA-CHALUFOUR, P. CORNILLET, P. LEHOANG, H. BETUEL, J.H.M. COHEN. HLA-A29 are sub-types and Choroidoretinopathy susceptibility : a possible "resistance motif" in the HLA-A29.1 molecule. *C.R. Acad. Sci. Paris*, 313, Série III, 599-605, (1991).

-PRI-30- D. STOPPA-LYONNET, C. DUPONCHEL, T. MEO, J. LAURENT, Ph.E. CARTER, M. ARALA-CHAVES, J.H.M. COHEN, G. DEWALD, J. GOETZ, G. HAUPTMANN, G. LAGRUE, Ph. LESAVRE, M. LOPEZ-TRASCASA, G. MISIANO, C. MORAINÉ, A. SOBEL, P.J. SPATH, and M. TOSI. Recombinational biases in the rearranged C1-Inhibitor genes of hereditary angioedema patients. *Am. J. Hum. Genet.*, 49, 1055-62, (1991).

-PRI-31- P. LEHOANG, N. OZDEMIR, A. BENHAMOU, T. TABARY, C. EDELSON, H. BETUEL, R. SEMIGLIA, J.H.M. COHEN. HLA-A29.2 subtype associated with Birdshot retinochoroidopathy. *Am. J. Ophtalmol.*, 113, 33-5, (1992).

-PRI-32- J.H.M. COHEN, H.U. LUTZ, J.L. PENNAFORTE, A. BOUCHARD and M. KAZATCHKINE. Peripheral catabolism of CR1 (The C3b receptor, CD35) on erythrocytes from healthy individuals and patients with Systemic Lupus Erythematosus (SLE). *Clin. Exp. Immunol.*, 87, 422-428, (1992).

-PRI-33- T. TABARY, J. LI-YA and J.H.M. COHEN. Homogeneous phase pyrophosphate (PPi) measurement (H3PIM) A non radioactive, quantitative detection system for nucleic acid

specific hybridization methodologies including gene amplification. J. Immunol. Methods, 156, 55-60, (1992).

-PRI-34- P. CORNILLET, Ph. GREDY, J.L. PENNAFORTE, O. MEYER, F. PHILBERT, M.D. KAZATCHKINE, and JHM COHEN. Increased frequency of the long (S) allotype of CR1 (The C3b/C4b receptor, CD35) in patients with systemic lupus erythematosus. Clin. Exp. Immunol., 89, 22-25, (1992).

-PRI-35- F. BOUGY, V. NEFF-CONTINANT, C. FESSARD and J.H.M. COHEN. Discordance in the determination of non-or low-responders to HBV vaccine using IMX-AUSABR or AUSABR - RIA. Vaccine, 11, 489, (1993).

-PRI-36- J.H.M. COHEN. Pregnancy in elderly women. The Lancet, 348, 1668, (1993).

-PRI-37- P. CORNILLET, J.L. PENNAFORTE, F. PHILBERT, P. BOURGEOIS, M.F. KAHN, M.D. KAZATCHKINE, and JHM COHEN. Complement C4 gene deletion in patients with systemic Lupus Erythematosus in France. J. Rheumatol., 20, 1633-1634, (1993).

-PRI-38- J.H.M. COHEN. Working time span internal clock. Lancet, 342, 611, (1993).

-PRI-39- J.H.M. COHEN. SAS to save public Mail Service. Lancet, 343, 366, (1994).

-PRI-40- B-N. PHAM, J-F. MOSNIER, F. WALKER, C. NJAPOUM, F. BOUGY, C. DEGOTT, S. ERLINGER, J.H.M. COHEN, F. DEGOS. Flow cytometry CD4+/CD8+ ratio of liver-derived lymphocytes correlates with viral replication in chronic Hepatitis B. Clin. Exp. Immunol., 97, 403-410, (1994).

-PRI-41- J.H.M. COHEN. Gresham's law. Lancet, 344, 966, (1994).

-PRI-42- P. CHARNEAU, A.M. BORMAN, C. QUILLET, D. GUETARD, S. CHAMARET, J. COHEN, G. REMY, L. MONTAGNIER, F. CLAVEL. Isolation and Envelope Sequence of a Highly Divergent HIV-1 Isolate : Definition of a New HIV-1 Group. Virology, 205, 247-253 (1994).

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UNITED STATES PATENT APPLICATION.

OF

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FOR

IMMUNE COMPLEXES COMPRISING PROTEINS OF HUMAN
IMMUNODEFICIENCY VIRUS TYPE 2 (HIV-2), ANTIBODIES
AGAINST PROTEINS OF HIV-2, METHODS AND KITS
EMPLOYING PROTEINS OF HIV-2, AND IMMUNOGENIC
COMPOSITIONS COMPRISING PROTEINS OF HIV-2

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Serial No. 08/002,756, filed January 13, 1993 (pending), which is a division of application Serial No. 07/356,459, filed May 25, 1989, now U.S. Patent No. 5,208,321. Serial No. 08/002,756 is also a continuation-in-part of application Serial No. 07/204,346, filed June 9, 1988 (abandoned), which is a continuation application of Serial No. 07/804,712, filed December 6, 1991, now U.S. Patent No. 5,312,902. The related applications are specifically incorporated by reference.

BACKGROUND OF THE INVENTION

This invention relates to viral proteins and glycoproteins, to compositions containing these proteins, to methods of preparing the proteins, and to their use in detecting viral infection.

The etiological agent of acquired immunodeficiency syndrome (AIDS) is the retrovirus referred to as human immunodeficiency virus (HIV) (Montagnier et al., 1984). To date, two related but distinct viruses, HIV-1 and HIV-2, have been identified (Barre-Sinoussi et al., 1983; Popovic et al., 1984; Levy et al., 1984; Wain-Hobson et al., 1985a; Clavel et al., 1986a; Brun-Vezinet et al., 1987; Guyader et al., 1987). HIV-2 is closely related to simian immunodeficiency virus (SIV), which causes an AIDS-like disease in macaques (Daniel et al., 1985; Sonigo et al., 1985; Chakrabarti et al., 1987).

HIV-1, HIV-2, and SIV show all the features of retrovirus family members (Wain-Hobson et al., 1985b; Montagnier and Alizon, 1987; Guyader et al., 1987; Chakrabarti et al., 1987). Their proviral genomes contain two long terminal repeats (LTRs) and three essential genes required for virus replication encoding the viral internal structural proteins (*gag*), the reverse transcriptase (*pol*), and the envelope glycoproteins (*env*) of the virus. In addition to these genes, both HIVs and SIV contain additional genes encoding the proteins that regulate viral expression (*tat* and *art/trs*) and three other genes encoding proteins of unknown function (*Q* or *sor*, *F* or *3'orf*, and *R*). The only notable difference in the genetic organizations of HIV-1, HIV-2, and SIV resides in the open reading frame referred to as *X*, which is absent in HIV-1.

Alignments of the nucleotide sequences of HIV-1, HIV-2, and SIV reveal a considerable homology between HIV-2 and SIV. These two viruses share about 75% overall nucleotide sequence homology, but both of them are only distantly related to HIV-1 with about 40% overall homology (Guyader et al., 1987; Chakrabarti et al., 1987). At the protein level, the *gag* and *pol* proteins of HIV-1, HIV-2, and SIV are antigenically cross-reactive, whereas *env* proteins are cross-reactive only between HIV-2 and SIV (Clavel et al., 1986b, 1987).

HIV-1, HIV-2, and SIV are both tropic and cytopathic for CD4 positive T lymphocytes (Dagleish et al., 1984; Klatzman et al., 1984; McDougal et al., 1985; Clavel et al., 1986b, 1987; Kannagi et al., 1985; Fultz et al., 1986). A great number of studies have indicated that CD4 functions as the cellular receptor for HIV-1 (for references see Weiss, 1988).

The HIV-1 env gene codes for a 160Kd glycoprotein that is proteolytically cleaved to yield the extracellular and transmembrane proteins, gp120 and gp41, respectively (Montagnier et al., 1985). It has been demonstrated that HIV-1 recognition of CD4 is mediated by gp120. This complex gp120-CD4 can be identified by co-immunoprecipitation using antibodies specific for the CD4 antigen (McDougal et al., 1986). Following the binding of gp120 to CD4, the entry of HIV-1 into the cell might occur by viral envelope cell membrane fusion (Lifson et al., 1986; Sodroski et al., 1986; Stein et al., 1987; McClure et al., 1988). A putative fusogenic domain in gp41 (Kowalski et al., 1987), which has a sequence homologous to other fusion peptides (Phe-Leu-Gly; Gallaher, 1987), might provide at least one HIV fusion site necessary for this process (Marsh and Dagleish, 1988).

In the case of HIV-2, a high molecular weight protein of about 130Kd to about 140Kd has been associated with the major envelope glycoprotein (Clavel et al., Science, 233:343-346, 1986). Another glycoprotein having a molecular weight of 120Kd has been associated with the external glycoprotein of HIV-2 (Guyader et al., Nature, 362:662-669, 1987). Nevertheless, detailed information for HIV-2 envelope proteins and glycoproteins and their cleavage products and precursors is lacking.

There exists a need in the art for additional information on the structure and in vivo processing of HIV-2 proteins, and especially HIV-2 envelope proteins and glycoproteins. Such information would aid in identifying HIV-2 infection in individuals. In addition, such findings could aid in elucidating the mechanism by which HIV-2 infection and virus proliferation occur and thereby make it possible to devise modes of intervening in viral processes.

SUMMARY OF THE INVENTION

This invention aids in fulfilling these needs in the art by providing HIV-2 envelope proteins and glycoproteins in purified form. Four glycoproteins of apparent molecular weights 300,000, 140,000, 125,000, and 36,000 daltons (gp300, gp140, gp125, and gp36) are detectable in human immunodeficiency virus type 2 (HIV-2) infected cells. The gp125 and gp36 are the external and transmembrane components, respectively, of the envelope glycoproteins of HIV-2 mature virions. It has now been

discovered that the gp300 is a dimeric form of gp140, which is the precursor of HIV-2 envelope glycoprotein. This invention thus provides gp300 glycoprotein of HIV-2 and human retroviral variants of HIV-2 in purified form.

This invention also provides proteins of HIV-2 or of a human retroviral variant of HIV-2 having apparent molecular weights of about 200Kd (p200) and about 90 to about 80 Kd (p90/80). These proteins are substantially unglycosylated and are in a purified form.

A similar high molecular weight glycoprotein of Simian Immunodeficiency Virus (SIV) or of a Simian retroviral variant of SIV has also been discovered. This glycoprotein is a precursor of an envelope glycoprotein of SIV and has an apparent molecular weight of about 300Kd (gp300_{SIV}). This glycoprotein is also provided in a purified form.

This invention also provides labeled gp300 of HIV-2 and gp300 of SIV. Preferably, the labeled glycoproteins are in purified form. It is also preferred that the labeled glycoprotein is capable of being immunologically recognized by human body fluid containing antibodies to HIV-2 or SIV. The gp300 glycoproteins can be labeled, for example, with an immunoassay label selected from the group consisting of radioactive, enzymatic, fluorescent, chemiluminescent labels, and chromophores.

Immunological complexes between the proteins and glycoproteins of the invention and antibodies recognizing the proteins and glycoproteins are also provided. The immunological complexes can be labeled with an immunoassay label selected from the group consisting of radioactive, enzymatic, fluorescent, chemiluminescent labels, and chromophores.

Furthermore, this invention provides a method for detecting infection of cells by human immunodeficiency virus type-2 (HIV-2). The method comprises providing a composition comprising cells suspected of being infected with HIV-2, disrupting cells in the composition to expose intracellular proteins, and assaying the exposed intracellular proteins for the presence of gp300 glycoprotein of HIV-2. The exposed intracellular proteins are typically assayed by electrophoresis or by immunoassay with antibodies that are immunologically reactive with gp300 glycoprotein of HIV-2.

This invention provides still another method of detecting antigens of HIV-2, which comprises providing a composition suspected of containing antigens of HIV-2, and assaying the composition for the presence of gp300 glycoprotein of HIV-2. The composition is typically free of cellular debris.

A method of distinguishing HIV-2 infection from HIV-1 infection in cells suspected of being infected therewith has also been discovered. The method comprises providing an extract containing intracellular proteins of the cells, and assaying the

extract for the presence of gp300 glycoprotein. The gp300 is characteristic of HIV-2, but the glycoprotein has not been found in extracts of HIV-1 cell cultures.

In addition, this invention provides a method of making gp300 glycoprotein of HIV-2, which comprises providing a composition containing cells in which HIV-2 is capable of replicating, infecting the cells with HIV-2, and culturing the cells under conditions to cause HIV-2 to proliferate. The cells are then disrupted to expose intracellular proteins. The gp300 glycoprotein is recovered from the resulting exposed intracellular proteins.

This invention also provides an *in vitro* diagnostic method for the detection of the presence or absence of antibodies which bind to an antigen comprising the proteins or glycoproteins of the invention or mixtures of the proteins and glycoproteins. The method comprises contacting the antigen with a biological fluid for a time and under conditions sufficient for the antigen and antibodies in the biological fluid to form an antigen-antibody complex, and then detecting the formation of the complex. The detecting step can further comprise measuring the formation of the antigen-antibody complex. The formation of the antigen-antibody complex is preferably measured by immunoassay based on Western Blot technique, ELISA (enzyme linked immunosorbent assay), indirect immunofluorescent assay, or immunoprecipitation assay.

A diagnostic kit for the detection of the presence or absence of antibodies, which bind to the proteins or glycoproteins of the invention or mixtures of the proteins and glycoproteins, contains antigen comprising the proteins, glycoproteins, or mixtures thereof and means for detecting the formation of immune complex between the antigen and antibodies. The antigens and the means are present in an amount sufficient to perform the detection.

Precursors of the envelope glycoproteins of HIV-2 and SIV can be prepared according to this invention. Specifically, this invention provides a method of preparing the precursors, which comprises providing an extracellular composition containing gp300 glycoprotein of HIV-2 or SIV at a pH of at least about 6.5. The pH of the composition is then lowered to a value of about 4 to about 6.0 in order to dissociate the gp300 glycoprotein into gp140 glycoprotein of HIV-2 or gp140 glycoprotein of SIV.

Finally, this invention provides an immunogenic composition comprising a protein or glycoprotein of the invention in an amount sufficient to induce an immunogenic response *in vivo*, in association with a pharmaceutically acceptable carrier therefor.

The proteins and glycoproteins of this invention are thus useful as a portion of a diagnostic composition for detecting the presence of antibodies to antigenic proteins associated with HIV-2 and SIV. In addition, the proteins and glycoproteins can be used to raise antibodies for detecting the presence of antigenic proteins associated with HIV-2 and SIV. The proteins

and glycoproteins of the invention can be also employed to raise neutralizing antibodies that either inactivate the virus, reduce the viability of the virus in vivo, or inhibit or prevent viral replication. The ability to elicit virus-neutralizing antibodies is especially important when the proteins and glycoproteins of the invention are used in vaccinating compositions.

BRIEF DESCRIPTION OF THE DRAWINGS

This invention will be described in greater detail by referring to the drawings in which:

Figure 1A is a fluorograph in which high molecular weight proteins of HIV-1 and HIV-2 are compared after electrophoresis in a polyacrylamide SDS gel;

Figure 1B depicts the result of electrophoresis of HIV-2 glycoproteins in an acrylamide gel;

Figure 2 depicts the result of two dimensional gel electrophoretic analysis of HIV-2 glycoproteins;

Figure 3(a) is a fluorograph of dissociated gp300 of HIV-2;

Figure 3(b) and Figure 3(c) are fluorographs of denatured gp300 of HIV-2;

Figure 4 shows the result of electrophoresis of HIV-2 glycoproteins after the glycoproteins were digested with beta-N-acetylglucosaminidase H (endo H);

Figure 5 is a fluorograph of a polyacrylamide gel after electrophoresis of HIV-2 glycoproteins, which were isotopically labeled with ^{14}C -mannose or ^3H fucose;

Figure 6 shows the result of electrophoresis of HIV-2 envelope proteins obtained from cultures in which N-linked glycosylation was inhibited by the antibiotic tunicamycin;

Figure 7 is a fluorograph of a polyacrylamide gel after electrophoresis of HIV-2 envelope glycoproteins obtained from cell cultures with and without oligosaccharide trimming inhibitors;

Figures 8A and 8B depict the results of electrophoresis of HIV-2 glycoproteins obtained during pulse-chase experiments in HIV-2 infected CEM cells in the absence (control) or presence of castanospermine (Fig. 8A) or monensin (Fig. 8B);

Figure 9 is a fluorograph of polyacrylamide gels after electrophoresis of SIV envelope glycoproteins labeled with ^{35}S -methionine, ^3H -fucose, or ^{14}C -mannose; and

Figure 10 is a schematic pathway postulated for *in vivo* processing of HIV-2 envelope glycoprotein.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

As a result of this invention, the processing of HIV-2 envelope glycoproteins has now been characterized. Four glycoproteins referred to as gp300, gp140, gp125, and gp36 are synthesized in HIV-2 infected cells. The gp125 and gp36 correspond to the external and transmembrane glycoproteins of HIV-2 virion, whereas gp300 and gp140 are only detectable in infected cells. The gp300 is a dimeric form of gp140, which is the immature precursor of HIV-2 envelope glycoprotein. This

dimer is very stable since it resists ionic and non-ionic detergents, high salt, 4M urea, and reducing agents. However, the dimer can be dissociated in acidic pH to yield gp140.

Dimerization occurs in the endoplasmic reticulum after the removal of glucose residues by glucosidases I and II, and after the action of Golgi mannosidases, the dimer becomes dissociated probably due to a shift in pH of the environment in trans Golgi. Finally, proteolytic cleavage of the mature precursor occurs outside the Golgi.

Transient dimer formation of the glycoprotein precursor seems to be an intrinsic property of the polypeptide moiety of HIV-2 envelope. This is a novelty in the mechanism of glycoprotein processing with N-linked oligosaccharide chains. It is hypothesized that conformational modifications brought about by the formation of this dimer are necessary for transport of the glycoprotein precursor to the Golgi apparatus.

I. IDENTIFICATION OF THE HIV-2 ENVELOPE GLYCOPROTEINS

Recently, it has been reported that the envelope gene of HIV-2 (ROD isolate) encodes a precursor glycoprotein that is then cleaved proteolytically to yield a 120Kd extracellular glycoprotein and a 36Kd transmembrane glycoprotein (Clavel et al., 1986a and 1986b). To identify the precursors of the HIV-2 glycoproteins, viral proteins in infected cells as well as in virus particles were studied. For comparison, the synthesis of

HIV-1 proteins in cells infected with HIV-1 (BRU isolate) were also studied. The results are shown in Figure 1 and were obtained as follows.

A. Comparison of high molecular weight proteins of HIV-1 and HIV-2 (FIGURE 1A)

CEM cells infected with HIV-1 or HIV-2 were labeled with ^{35}S -methionine (200 $\mu\text{Ci/ml}$; 4×10^6 cells/ml) for 18 hours. Extracts from these infected cells (CELL) and their corresponding culture medium (SN) were purified on specific immunoaffinity columns:

HIV-1 serum-Sepharose specific for HIV-1 proteins (Krust et al., 1988), and

HIV-2 serum-Sepharose specific for HIV-2 proteins.

(See "Experimental Procedures").

These purified proteins were analyzed by electrophoresis in a 7.5% polyacrylamide SDS-gel containing 6M urea. A fluorograph of the gel is presented in Figure 1. The sizes of the HIV-1 and HIV-2 proteins are indicated on the left and right of the lanes shown in Figure 1.

Referring to Figure 1A, the p68 and p55 are the reverse transcriptase and the gag precursor, respectively. The gp160 and gp120 are the glycoprotein precursor of HIV-1 envelope and its cleaved product.

Three major high molecular weight glycoproteins of 300, 140, and 125Kd are detectable in HIV-2 infected cells (Figure 1A). The proteins are specific to HIV-2 because they are absent in non- infected cells and because they could be consistently identified by all HIV-2, but not HIV-1, seropositive sera in an immunoprecipitation assay (data not shown).

In side by side comparison, the electrophoretic mobility of these three HIV-2 proteins is clearly different from that of the 160Kd HIV-1 precursor glycoprotein (gp160) and one of its cleaved products, 120Kd external envelope glycoprotein (gp120; Figure 1A). It should be noted that the resolution of the 140 and 125Kd proteins of HIV-2 from one another can be clearly observed in polyacrylamide-SDS gels containing a high concentration of urea. In the absence of urea, these proteins migrate as a thick band. The 300 and 140Kd proteins are only detectable in infected cells, whereas the 125Kd protein is detectable both in infected cells as well as in the virus (Figure 1A).

B. Identification of HIV-2 glycoproteins (FIGURE 1B)

The glycosylation of the 300, 140, and 125Kd proteins was demonstrated by metabolic labeling with ³H-glucosamine (Figure 1B). More particularly, HIV-2 CEM cells and

T-lymphocytes were labeled with ^3H -glucosamine (200 $\mu\text{Ci/ml}$; 4×10^6 cells/ml) for 18 hours. Extracts from infected cells (lanes C) and culture medium containing virus (lanes V) were purified on the HIV-2 serum-Sepharose column. The labeled proteins were analyzed by electrophoresis in a 7.5% gel. The lane on the far left depicts the result of electrophoresis in a 12.5% acrylamide gel and shows the presence of gp36. The gp36 is only slightly glycosylated and its detection required longer exposure times. Specifically, this part of the Figure had to be overexposed to see gp36; for this reason gp140/gp125 are resolved as a thick band.

The presence of gp300 and gp140 is not restricted to infected CEM cells. They are also detectable in HIV-2 infected T4 lymphocytes as depicted in Figure 1B. As in CEM cell cultures, gp300 and gp140 are detectable only in infected cells, whereas gp125 is present both in cells and in HIV-2 particles.

These results indicate that among the glycoproteins detectable in HIV-2 infected cells, gp125 and gp36 correspond to the virion envelope, whereas gp300 and gp140 might be precursors of the envelope glycoproteins.

C. Characterization of gp300 and gp140 (FIGURE 2)

The proteins gp300, gp140, and gp125 were labeled with ^{35}S -methionine and analyzed by two dimensional gel electrophoresis. The patterns of resolution that were obtained indicated that gp300 and gp140 are closely related.

More particularly, ^{35}S -methionine labeled gp300, gp140, and gp125 purified from HIV-2 infected CEM cells (CELL) and culture medium containing virus (SN) prepared in the same manner as the experiments reported in Figure 1 were analyzed by two dimensional gel electrophoresis (See "Experimental Procedures"). The pH gradient obtained by isoelectric focusing (first dimension) is shown in Figure 2. In the second dimension, proteins were resolved on a 7.5% polyacrylamide-SDS gel containing 6M urea. Fluorographs of the gels are presented in Figure 2.

Both proteins were resolved as an heterogeneous subspecies with identical isoelectric points (pI) in the pH range of 6.8 to 7.8 (Figure 2). This similarity between gp140 and gp300 suggested that gp300 is a dimeric form of gp140 (see below).

The gp125, which is present in both infected cells and in virus particles, exhibited less heterogeneity and migrated with pI values between 6.2 to 6.5. In infected cells, there was a minor subspecies of gp125 with a pI value of 7.2 to 7.3. This basic gp125 is not incorporated into the HIV-2 virion. Thus it might represent a glycoprotein that is not processed properly. The acidic nature of the mature gp125 might be due to the addition of sialic acid on some of its carbohydrate side chains during the processing of the envelope glycoprotein.

D. Dissociation of the native (a) and the denatured (b and c) gp300 (FIGURE 3)

The gp300 is very stable since it resists ionic (1% SDS) and non-ionic (2% Triton X-100) detergents, urea (2-6M), high

salt (1M NaCl), and reducing agents (1% β -mercaptoethanol). However, it was possible to demonstrate that gp300 could be dissociated into gp140 in acidic pH. In these experiments, immunoaffinity column bound proteins were incubated in acetate buffer at pH values varying between 4 to 7. These samples were then analyzed by polyacrylamide gel electrophoresis. Fluorographs of the gels are shown in (a), (b), and (c) in Figure 3. In section (c), the band of gp300 and the dissociated gp140 were quantified by densitometric scanning of the fluorograph. More particularly, the gels were prepared as follows.

(a) ^{35}S -methionine labeled extracts from HIV-2 infected CEM cells were purified on the HIV-2 serum-Sepharose column. This sample was then divided into two equal aliquots: one was incubated in the binding buffer, Figure 3(a), lane 1, whereas the other one was incubated in buffer containing 30mM sodium acetate pH 4.0, 0.2mM PMSF, 100 units/ml aprotinin and 5mM β -mercaptoethanol, Figure 3(a), lane 2. After 1 hour at 37°C, the acidic medium was neutralized and both samples were analyzed by electrophoresis.

(b) Purified and lyophilized ^{35}S -methionine labeled gp300 was suspended in 100 μl of the sodium acetate buffer pH 4.0 as above Figure 3(b), lane 2. Incubations were carried out for 30 minutes at 37°C before addition of 2-fold electrophoresis sample

buffer containing 2M urea. In lane 1 of Figure 3(b), the lyophilized gp300 was directly suspended in the electrophoresis sample buffer.

(c) The purified and lyophilized ^{35}S -methionine labeled gp300 was suspended in solution containing 30mM Tris-HCl, 0.2mM PMSF and 100 units/ml aprotinin and buffered with HCl at pH 7.5, 7.0, 6.5 and 6.0 (as indicated). After 60 minutes at 37°C, two fold electrophoresis sample buffer was added and the samples were analyzed by electrophoresis.

Figure 3(a) shows that the band of gp300 shifted to the position of gp140 when the sample was incubated at pH 4. Further experiments were carried out using purified preparations of gp300 obtained by preparative gel electrophoresis. Such denatured samples of gp300 were dissociated completely in acetate buffer at pH 6.0, Figure 3(b). The efficiency of dissociation of the purified gp300 was probably due to a decrease in the pH along with the presence of residual SDS in the lyophilized sample, since column-bound native gp300 did not dissociate in the same buffer at pH values higher than pH 5 (data not shown).

In Tris-HCl buffer, the dissociation was less efficient. At pH 7.5 there was only a slight dissociation of gp300 to gp140, but it increased with decreasing pH values. In Tris buffer at pH 6.0, the dissociation was about 80%, (Figure 3(c)).

During dissociation of the pure gp300 in either acetate or Tris-HCl buffer no proteins other than gp140 were detectable (experiments carried out in 15% polyacrylamide gels; data not shown).

These results indicate that gp300 is a dimeric form of gp140, the precursor of HIV-2 envelope glycoprotein. Thus, it seems most likely that during the processing of the envelope glycoprotein, two molecules of gp140 become fused by a pH-dependent mechanism.

E. Characterization of the Oligosaccharide Side Chains of HIV-2 Glycoproteins (FIGURES 4 & 5)

Digestion with endo β -N-acetylglucosaminidase H (endo H) demonstrated the presence of N-linked oligosaccharides of the high mannose type on HIV-2 glycoproteins. The gp300, the gp140 + gp125, and the gp125 were purified by immunoaffinity chromatography and preparative electrophoresis. (See "Experimental Procedures"). The lyophilized samples were suspended in endo H digestion buffer, which does not promote the dissociation of gp300 to gp140. The procedure was carried out as follows.

Purified and lyophilized gp300, gp140/gp125, and gp125 ("Experimental Procedures") were suspended in buffer containing 150mM sodium citrate pH 5.5, 0.1% SDS (w/v), 0.5mM PMSF before heating for 2 minutes at 90°C. Aliquots of these samples were then incubated (2 hours, 30°C) without (lane 1, Fig. 4), or with 0.4 milli-units of endo-H (lane 2, Fig. 4), 2 milli-units of endo-H (lane 3, Fig. 4), and 10 milli-units of endo-H (lane 4,

Fig. 4). All the reactions were stopped by the addition of two fold electrophoresis sample buffer. Electrophoresis was as previously described in relation to Figure 1. Fluorographs of the different gels are shown in Figure 4. The arrows p90 and p80 on the right indicate the position of the digested product. Conditions for endo-H digestion were as described (Tarentino et al., 1974).

Upon endo H digestion, the electrophoretic mobility of gp300 was reduced to a protein of 200-250Kd. A small fraction of gp300 that had become dissociated into gp140, was digested to give rise to a 80Kd protein (Figure 4, section gp300, lane 4).

The gp140 + gp125 sample was digested by endo H into 90 and 80Kd proteins whereas gp125 was converted into a 90Kd protein (Figure 4, sections gp140/125 and gp125). These results indicate that endo H digestion of gp140 and gp125 give products of molecular weight 80 and 90Kd, respectively. The resistance to endo H digestion of gp125 relative to gp140 is probably due to the conversion of some high mannose type oligosaccharide side chains into complex oligosaccharides during processing of the envelope glycoprotein (Kornfeld and Kornfeld, 1985).

Metabolic labeling of cells was carried out with ^{14}C -mannose and ^3H -fucose. More particularly, HIV-2 infected CEM cells were labeled (18 hours) with ^{14}C -mannose 25 $\mu\text{Ci/ml}$; 4×10^6 cells/ml) or ^3H -fucose (200 $\mu\text{Ci/ml}$; 4×10^6 cells/ml). Extracts from infected cells (lanes C, Fig. 5) and culture

medium containing virus (lanes V, Fig. 5) were purified on HIV-2 serum-Sepharose. Labeled glycoproteins were then analyzed by polyacrylamide gel electrophoresis. A fluorograph is shown in Figure 5.

Referring to Figure 5, it will be apparent that metabolic labeling resulted in the incorporation of mannose into gp300, gp140, and gp125 whereas only gp300 and gp125 were able to incorporate fucose. Fucose residues are normally transferred on oligosaccharide chains late in the glycosylation cycle, after the action of trimming enzymes of the endoplasmic reticulum and Golgi apparatus (Kornfeld and Kornfeld, 1985; Fuhrmann et al., 1985). The fact that gp140 does not contain fucose residues was consistent with it being the precursor of gp300 and gp125.

F. The Effect of Glycosylation Inhibitor
Tunicamycin on the Processing of HIV-2
Glycoproteins (FIGURE 6)

All glycoproteins carrying N-linked glycans derive their oligosaccharide moiety from the lipid-linked oligosaccharide, $\text{Glc}_3\text{Man}_9\text{-GlcNAc}_2\text{-pp-Dolichol}$, through a reaction carried out by protein-oligosaccharidyl transferase, which catalyzes the en bloc transfer of oligosaccharide chains to asparagine residues (for references, see Kornfeld and Kornfeld, 1985). Tunicamycin blocks such N-linked glycosylation since it inhibits the production of N-acetylglucosamine pyrophosphoryldolichol, the first step in the assembly of lipid-linked oligosaccharides (Li et al, 1978; Heifetz et al., 1979).

In the presence of 2 $\mu\text{g/ml}$ tunicamycin, the overall N-linked glycosylation of HIV-2 envelope glycoproteins was completely blocked in infected CEM cells. This was demonstrated by the lack of ^3H -glucosamine incorporation in viral glycoproteins, gp300, gp140, and gp125. Inhibition of N-linked glycosylation by tunicamycin was carried out as follows.

HIV-2 infected cells in the absence (lanes C, Fig. 6) or presence (TM, Fig. 6) of tunicamycin (2 $\mu\text{g/ml}$) were labeled with ^{35}S -methionine (panel " ^{35}S -met"; 200 $\mu\text{Ci/ml}$; 4×10^6 cells/ml) or with ^3H -glucosamine (panel " ^3H -GlcNAc"; 200 $\mu\text{Ci/ml}$; 4×10^6 cells/ml) for 16 hours. Cells treated with tunicamycin were first incubated (37°C) with the antibiotic (2 $\mu\text{g/ml}$) for 2 hours before labeling with ^{35}S -methionine or ^3H -glucosamine. Extracts from infected cells (CELL) and from the culture medium containing virus (SN) were purified by HIV-2 serum-Sepharose and analyzed by polyacrylamide 7.5% gel electrophoresis. Fluorographs of the gels are presented in Fig. 6. The position of the unglycosylated envelope precursor (p90/80) and the unglycosylated dimer (200Kd) are indicated by the small arrows on the right. These 90/80Kd and 200Kd proteins do not incorporate ^3H -glucosamine (panel ^3H -GlcNAc, cell lane TM).

Under these experimental conditions, protein synthesis was not affected in infected cells treated with tunicamycin (data not shown). Such cultures isotopically labeled with ^{35}S -methionine accumulated two major proteins of apparent sizes 200 and 80-90Kd, which migrated as wide bands (Figure 6). The molecular weights of these proteins coincide well with endo H digestion products of gp300, gp140, and gp125 (Figure 4), thus suggesting that the 200 and 80-90Kd proteins correspond to unglycosylated forms of HIV-2 envelope glycoproteins. The molecular weight of the 80-90Kd protein corresponds to the expected molecular weight of unglycosylated HIV-2 envelope precursor estimated from its nucleic acid sequence (Guyader et al., 1987). The 200Kd protein is probably the dimeric form of the unglycosylated envelope precursor. These results confirm that HIV-2 envelope proteins have N-linked polysaccharide chains.

Besides inhibition of glycosylation, tunicamycin treatment inhibits the processing and export of the envelope glycoprotein since the 80-90Kd protein was not found in the extracellular medium (Figure 6, lanes SN). Oligosaccharide chains of HIV-2 envelope proteins, therefore, are probably involved in the cellular transport through the Golgi apparatus. The absence of unglycosylated forms of the envelope protein in the extracellular medium of tunicamycin treated cells might also be due to its rapid degradation. Several reports have suggested

that the unglycosylated form of a protein is more sensitive to proteases than its glycosylated form (Olden et al., 1978; Schwartz et al., 1976). Accordingly, the small molecular weight proteins in ^{35}S -methionine labeled cells cultured with tunicamycin might represent partially degraded products of the unglycosylated envelope protein (Figure 6).

G. Effect of Oligosaccharide Trimming Inhibitors on the Synthesis of HIV-2 Glycoproteins (FIGURE 7)

Asparagine-linked oligosaccharides ($\text{Glc}_3\text{Man}_q\text{GlcNAc}_2$) of glycoproteins undergo extensive modifications or processing following their attachment to nascent proteins (reviewed by Kornfeld and Kornfeld, 1985). The trimming reactions occur in the lumen of the rough endoplasmic reticulum (RER) and in the Golgi apparatus by specific glucosidases and mannosidases.

Processing of oligosaccharide chains of glycoproteins can be manipulated with the aid of specific inhibitors of the trimming glucosidases and mannosidases (reviewed by Schwarz and Datema, 1984; Fuhrmann et al., 1985). In these experiments, different trimming inhibitors were used to investigate the localization of HIV-2 glycoprotein precursors and also to study the role of glycosylation in the processing of the envelope precursor. The inhibitors used were:

castanospermine, a plant alkaloid that inhibits glucosidase I (Saul et al., 1983);

deoxynojirimycin (dNM), a glucose analogue that inhibits trimming glucosidase I and II (Lemansky et al., 1984);

1-deoxymannojirimycin (dMM), a mannose analogue that inhibits mannosidase catalyzed reactions (Fuhrmann et al., 1984);

bromoconduritol (6-bromo-3,4,5-trihydroxycyclohex-1-ene) that inhibits glucosidase II (Datema et al., 1982); and

swainsonine, an indolizidine alkaloid that inhibits Golgi mannosidase II (Tulsiani et al., 1982).

Specifically, HIV-2 infected CEM cells were labeled (16 hours, 37°C) with ³⁵S-methionine (200 µCi/ml; 4 x 10⁶ cells/ml) in the absence (lanes T, Fig. 7) or presence of the oligosaccharide trimming inhibitor

1mM bromoconduritol (lanes Bro, Fig. 7);

1mM castanospermine (lanes Cast, Fig. 7);

10 µg/ml swainsonine (lanes Sw, Fig. 7);

3mM deoxynojirimycin (lanes dNM, Fig. 7); and

1mM deoxymannojirimycin (lanes dMM, Fig. 7).

Extracts from infected cells (panel CELL) and from culture medium containing virus particles (panel SN) were purified on HIV-2 serum-Sepharose to identify viral glycoproteins gp125, gp140, and gp300 in infected cells and gp125 in culture medium. All samples were analyzed by polyacrylamide (7.5%) gel electrophoresis.

In order to show that inhibition of gp125 production by cells treated with different inhibitors is specific to the viral glycoprotein, culture media were assayed for viral core protein p26 by an immunoprecipitation assay using an HIV-2 seropositive

serum (Clavel et al., 1986a, 1987). The p26 was analyzed by polyacrylamide (12.5%) gel electrophoresis. Figure 7 represents a fluorograph showing only one part of each gel.

As expected, control infected cells contained gp300, gp140, and gp125 whereas only gp125 was observed in the extracellular medium (Figure 7, sections cell and SN, lanes T). In cells treated with castanospermine or dNM, there was a normal level of gp300, no gp125 and a small amount of a 150Kd protein that probably corresponds to the glucosylated form of gp140. In such cells, therefore, the processing of the envelope glycoprotein was blocked since no gp125 was detectable in the extracellular medium in spite of the production of p26, the core protein of HIV-2 (Figure 7, lanes Cast and dNM). These results indicate that removal of the terminal glucose residues from the oligosaccharide chains of the envelope glycoprotein precursor is necessary for its processing and cleavage by the cellular protease.

Bromoconduritol, which acts on glucosidase II, also inhibited by 70-90% the normal production of gp125, but the levels of gp140 and gp300 remained normal (Figure 7, lanes Bro). In contrast to castanospermine and dNM (which inhibit removal of terminal glucose residue), bromoconduritol treatment (which inhibits removal of two inner glucose residues) did not block completely the processing of HIV-2 envelope glycoprotein. In fact, low amounts of gp125 were detectable intracellularly and extracellularly. This latter result suggests that a low level

of mannose trimming can occur without removal of the two inner glucose residues. Such a phenomenon has been observed previously for the processing of other viral glycoproteins during bromoconduritol treatment (Datema et al., 1982).

Mannosidase inhibitors, swainsonine and dMM, did not cause an apparent modification in the level of intracellular gp300, gp140, and gp125, but the level of extracellular gp125 was 50% less than that from the corresponding control cells (Figure 7, lanes Sw and dMM). Thus, although the oligosaccharide chain was only deglycosylated, the glycoprotein precursor was proteolytically cleaved to yield a protein similar to gp125 but with a higher content of mannose, which probably affected the cellular transport of gp125. The molecular weight of the extracellular glycoprotein produced in the presence of dMM was slightly higher than that produced in the absence of the inhibitor. This is probably due to the higher content of mannose residues in the extracellular protein synthesized by dMM-treated cells (Figure 7, section SN).

It should be emphasized that the effects of trimming enzyme inhibitors on the processing of HIV-2 envelope glycoprotein were specific since the synthesis (data not shown) and the production of HIV-2 p26 was not affected at all (Figure 7, section SN).

H. Effect of Castanospermine and Monensin on the Processing of HIV-2 Glycoproteins (FIGURE 8)

To study the intracellular processing of HIV-2 glycoproteins, pulse-chase experiments were performed. The results are shown in Figure 8. More particularly, the experiments were carried out as follows:

(a) Pulse-chase experiments were performed in HIV-2 infected CEM cells in the absence (Control) or presence of 1mM castanospermine (Cast.). Control: infected cells were incubated-1 hour at 37°C in methionine-free medium before 15 minutes pulse labeling with ^{35}S -methionine (200 $\mu\text{Ci/ml}$; 4×10^5 cells/ml; lane 1, Fig. 8a). The radioactive label was then chased in culture medium containing 5mM cold methionine for 0.5, 1.5, and 3 hours (in lanes 2, 3, and 4, respectively, Fig. 8a). Cast.: HIV-2 infected CEM cells were incubated (1 hour, 37°C) in methionine-free medium containing castanospermine before 30 minutes pulse labeling with ^{35}S -methionine (lane 1, Fig. 8a). These cells were then chased as above, but in the presence of castanospermine for 0.5, 1.5, and 3 hours (lanes 2, 3, and 4, respectively, Fig. 8a).

The gp140 was the first protein detectable 15 minutes after pulse labeling. During the chase, gp300 became detectable at 0.5 hours, whereas gp125 became detectable at 1.5-3 hours. The fact that gp300 was observed after synthesis of gp140 and the fact that gp125 was detectable only after formation of gp300 (Figure 8A, lanes 1-4), suggest that dimerization is an intermediate step necessary for the oligosaccharide processing

towards the mature glycoprotein, gp125. This suggestion was confirmed by the use of castanospermine, which inhibits the trimming of the external glucose residue of polysaccharide chains.

After 30 minutes of pulse labeling in the presence of castanospermine, a 150Kd protein was detectable along with gp300 (Figure 8, Cast., lane 1). The 150Kd protein should correspond to gp140; the slight increase in the molecular weight of the first precursor is ascribed to the presence of glucose residues in its oligosaccharide chains. Thus, gp140 synthesized in HIV-2 infected cells represents the precursor glycoprotein without its glucose residues. Accordingly, the 150Kd protein (gp150) represents the first immature glycoprotein of HIV-2 envelope. The removal of glucose residues in control cells has been reported to be a rapid process occurring during or briefly after cotranslational translocation of precursor glycoproteins into endoplasmic reticulum (Lemansly et al., 1984). After 30 minutes of pulse and 3 hours of chase in the presence of castanospermine, the level of gp150 was gradually reduced while gp300 accumulated (Figure 8, Cast, lanes 1-4). Under these conditions, the precursor was not cleaved to yield gp125.

Further characterization of HIV-2 envelope glycoprotein was studied in pulse-chase experiments using monensin, a cationic ionophore that inhibits the transport of proteins from Golgi to the plasma membrane or in some cases it might even block the

transport of proteins at the level of the medial Golgi cisternae (Tartakoff and Vassali, 1977; Johnson and Schlesinger, 1980; Strous and Lodish, 1980; Griffiths et al., 1983). HIV-2 infected cells in the absence or presence of monensin were pulsed labeled as follows:

(b) Pulse chase experiments in HIV-2 infected cells were carried out in the absence (Control) or presence of 1 μ M monensin. Infected cells with or without monensin were incubated (1 hour, 37°C) in methionine-free medium before 30 minutes pulse labeling with 35 S-methionine (lanes 1, Fig. 8b). Labeled cells were then chased in culture medium containing 5mM cold methionine for 0.5, 1.5, and 3 hours (lanes 2, 3, and 4, respectively, Fig. 8b). Extracts were purified on HIV-2 serum-Sepharose, and labeled proteins were analyzed by polyacrylamide (7.5%) gel electrophoresis. Fluorographs are shown in Figure 8b. (The p55 shows the gag precursor in section A, lanes 1.)

In the presence of monensin, HIV-2 infected cells synthesized normal levels of gp140 and its dimeric form. However, no gp125 was detectable in monensin treated cells. After 1.5-3 hours of chase, monensin treated cells accumulated a 135Kd protein (gp135) that is probably the dissociated product of the dimer precursor. The slightly smaller molecular weight of gp135 might be accounted for by the removal of some mannose residues by the action of RER and Golgi mannosidases. In view of these results, it is tempting to speculate that after

deglucosylation, gp300 becomes trimmed by mannosidases of RER and Golgi before its dissociation into the mature precursor gp135 of HIV-2 envelope. This gp135 could then be transported to plasma membrane and also be cleaved by cellular protease. Inhibition of protein transport by monensin blocks the mature glycoprotein gp135 in trans Golgi. No mature envelope glycoproteins are detectable in monensin treated cells, intracellularly or extracellularly, although p26 is synthesized and excreted (data not shown).

I. Dimerization of the Glycoprotein
Precursor Occurs also in SIVmac Infected
Cells (FIGURE 9)

The nucleotide sequence of HIV-2 envelope shows a considerable homology (75% amino acid identity) to that of SIV (Guyader et al., 1987; Chakrabarti et al., 1987; Franchini et al., 1987). For this reason, it was important to investigate whether dimerization of envelope glycoproteins is detectable in SIV infected cells. SIV proteins were purified by the immunoaffinity column containing antibodies specific for HIV-2 proteins, since the gag, pol, and env proteins of HIV-2 and SIV are antigenically cross-reactive.

More particularly, SIV-infected HUT-78 cells were labeled (16 hours, 37°C) with ^{35}S -methionine (200 $\mu\text{Ci}/\mu\text{l}$; 4×10^6 cells/ml), ^3H -fucose (200 $\mu\text{Ci}/\mu\text{l}$; 4×10^6 cells/ml) and ^{14}C -mannose (25 $\mu\text{Ci}/\mu\text{l}$; 4×10^6 cells/ml). Extracts from infected cells (lanes C, Fig. 9) and from the culture medium

containing SIV (lanes V, Fig. 9) were purified on HIV-2 serum-Sepharose. Because of cross-reactivity between HIV-2 and SIV proteins, the HIV-2 positive serum could be used to immunoprecipitate SIV proteins. All samples were analyzed by polyacrylamide (7.5%) gel electrophoresis. (See "Experimental Procedures".) A fluorograph of the different gels is shown in Figure 9.

Figure 9 shows that SIV infected cells synthesize three high molecular weight proteins analogous to those synthesized in HIV-2 infected cells: gp300, gp140, and gp130. The electrophoretic mobility of gp300_{SIV} and gp140_{SIV} correspond to that of HIV-2 glycoproteins gp300 and gp140 (data not shown). The gp130_{SIV} has a slightly higher mobility than gp125 of HIV-2. The p55 labeled with ³⁵S-methionine is probably the gag precursor of SIV.

Evidence that these proteins present in SIV infected cells are glycoproteins was provided by the isotopic labeling with ¹⁴C-mannose and ³H-fucose. All the three proteins incorporated mannose, but only gp300_{SIV} and gp130_{SIV} incorporated fucose (Figure 9). The gp300_{SIV} and gp140_{SIV} are intracellular proteins, whereas gp130_{SIV} is the extracellular glycoprotein. The fact that gp300_{SIV} and gp130_{SIV} can incorporate fucose suggests that they are processed products of gp140_{SIV}.

These results indicate that doublet formation of the envelope glycoprotein precursor is a specific property of HIV-2 and SIV envelope gene expression. It should be emphasized that HIV-1 envelope glycoprotein does not undergo dimerization during its processing. HIV-1 infected cells in the presence of castanospermine or dNM do not accumulate envelope dimers (data not shown) as it is the case for HIV-2 or SIV.

* * *

This invention thus describes for the first time the processing of HIV-2 envelope glycoproteins and details a novel mechanism of glycoprotein processing with N-linked oligosaccharide chains. The envelope glycoproteins of HIV-2, i.e. the extracellular gp125 and transmembrane gp36, arise from a common precursor glycoprotein (Guyader et al., 1987). The unusual feature of this glycoprotein precursor is that it requires the formation of a homologous dimer in order to become transported and processed through the Golgi apparatus. The mechanism of dimerization of envelope glycoprotein is not entirely clear. The fact that the purified dimer can be dissociated at an acidic pH (pH 6.0) suggests that dimerization might be pH dependent. Oligosaccharide chains on the precursor glycoprotein are not essential for dimer formation. Evidence for this has been obtained by two different experiments: (1) Digestion with endo H results in a shift in the electrophoretic mobility of the dimer without dissociating it; and (2) In the

presence of tunicamycin, HIV-2 infected cells synthesize an unglycosylated envelope precursor (80-90Kd) that can form a dimer (200Kd). These results emphasize that the dimer formation is an intrinsic property of the polypeptide moiety of the envelope precursor.

Pulse-chase experiments in the absence or presence of castanospermine (Figure 8) suggest that dimerization of the glycoprotein precursor normally occurs immediately after removal of glucose residues. Since glucosidases are associated with membranes of endoplasmic reticulum, then it is most likely that dimerization occurs in the RER. In the presence of castanospermine, the dimer becomes accumulated in RER and it is not processed. However, once the glucose residues are removed, then inhibition of the RER mannosidase does not prevent the processing of the glycoprotein-dimer through the Golgi apparatus (Figure 7). Accordingly, the glucose residues in the oligosaccharide chains of the dimer precursor prevent its exit from the RER. In accord with this, it has been postulated that glucose trimming is necessary for efficient transport from the RER to the Golgi, possibly because the deglycosylated oligosaccharide forms part of a recognition site for a transport receptor (Lodish and Kong, 1984; Lemansky et al., 1984). It might also be possible that glucose removal is crucial for the precursor dimer to achieve a correct functional configuration (Schlesinger et al., 1984) that favors the action of trimming mannosidases.

In view of these results, a schematic pathway for the processing of HIV-2 envelope glycoproteins is proposed in Figure 10. With reference to Figure 10, the expected size of the polypeptide moiety of the precursor envelope glycoprotein is about 80Kd (Figures 4 and 6). The oligosaccharide chain is transferred from dolichol-P-P to the newly synthesized envelope precursor (80Kd) probably at acceptor amino-acid asparagine residues (Kornfeld and Kornfeld, 1985). As depicted in Figure 10, tunicamycin inhibits assembly of dolichol-P-P glycan, and for this reason the 80Kd protein does not become glycosylated.

Addition of oligosaccharide chains to the 80Kd protein yields the first envelope glycoprotein precursor, gp150. This precursor might or might not exist as such in infected cells, since addition of polysaccharide chains and glucose trimming probably occurs during translation of the precursor. Whatever is the case, gp150 becomes rapidly deglycosylated to give gp140. At this stage, a difference in environment, perhaps of pH, would trigger dimer formation by the fusion of two gp140 molecules. The resulting gp300 can then be trimmed by the RER mannosidase and transported to the Golgi apparatus.

In the presence of castanospermine or dNM, gp150 becomes dimerized and is accumulated in the RER. This dimer is not processed because it is glucosylated. However, as long as the dimer is found in the deglycosylated form, it can be transported to the Golgi; inhibition of RER mannosidase by dMM does not block processing of the dimer precursor.

In the Golgi, gp300 traverses the different compartments probably by vesicular transport (Griffiths and Simons, 1986) during which the oligosaccharide chain is further trimmed by Golgi mannosidases before addition of other sugars such as fucose and sialic acid. Evidence for fucose incorporation has been obtained by isotopic labeling of gp300 with ³H-fucose. Evidence for sialic acid incorporation was obtained indirectly by digesting gp300 with neuraminidase, an enzyme that hydrolyzes terminal N-acetylneuraminic acid in various glycoproteins (Peyrieras et al., 1983). The gp300 of HIV-2 is susceptible to digestion with neuraminidase as evidenced by a significant decrease in the electrophoretic mobility of the dimer (data not shown). The results are consistent with the precursor keeping its dimeric form all through its processing in the Golgi cis, medial, and trans cisternae before its transport to the trans-Golgi network (TGN; Griffiths and Simons, 1986) where it dissociates due to a drop in the pH of this compartment.

The dissociated dimer yields glycoproteins (gp135) of slightly smaller molecular weight than the first detectable glycoprotein precursor (gp150-140). The gp135 could then be transported to plasma membrane and also be cleaved by the cellular protease to yield the mature glycoproteins of HIV-2 envelope, gp125, and gp36. Monensin most probably inhibits transport from the Golgi to the plasma membrane; for this reason gp135 accumulates in the Golgi.

It is well accepted that the Golgi apparatus is implicated in the mechanism of sorting secretory and plasma membrane proteins, which seems to take place in the last Golgi compartment referred to as TGN (Griffiths and Simons, 1986). This compartment on the trans side of the Golgi stack, previously has been referred to as Golgi endoplasmic reticulum lysosomes (GERL) and recently as post-Golgi vacuoles or the trans-most cisternae of the Golgi stack (Novikoff, 1976; Saraste and Kuismanen, 1984; Orci et al., 1987). Interestingly, the pH of the TGN has been considered to be mildly acidic, i.e., about pH 6 (Anderson and Pathak, 1985; Griffiths and Simons, 1986). The acidic pH in the TGN could then account for the dissociation of the processed dimer.

The results discussed here illustrate that the processing of the envelope glycoproteins of HIV-2 is a multistep process involving the synthesis of an immature precursor gp150-140, the intermediary dimer precursor gp300 and finally the mature precursor gp135. Despite their evolutionary relationship, HIV-1

and HIV-2 have found different mechanisms for the processing of their envelope glycoproteins. Whether or not these differences are involved in their pathogenesis is under investigation.

Following is a more detailed description of the experimental procedures used in this invention.

II. EXPERIMENTAL PROCEDURES

A. Materials

L-(³⁵S)Methionine (specific activity 1000 Ci/mmol, L-(6-³H) Fucose (specific activity: 45-70 Ci/mmol), D-(6-³H)Glucosamine (specific activity: 20-40 Ci/mmol and D-(U-¹⁴C)Mannose (specific activity: 200-300 mCi/mmol) were purchased from Amersham (Amersham, UK). Bromoconduritol, castanospermine, 1-deoxymannojirimycin (dMM), 1-deoxynojirimycin (dNM), swainsonine and tunicamycin were obtained from Boehringer-Mannheim (Mannheim, West Germany). Endo B-N-acetylglucosaminidase H was from Calbiochem (San Diego, USA). Ampholines were purchased from Pharmacia (Uppsala, Sweden).

B. Virus and Cells

HIV-1_{BRU} isolate of the human immunodeficiency virus type 1 (Montagnier et al., 1984), HIV-2_{ROD} isolate of the human immunodeficiency virus type 2 (Clavel et al., 1986a), and Simian immunodeficiency virus, SIVmac₁₄₂ (Daniel et al., 1985), were used in this study.

The different cell lines and human lymphocytes were cultured in suspension medium RPMI-1640 (GIBCO-BRL, Cergy-Pontoise, France) containing 10% (v/v) fetal calf serum; 2 µg/ml polybrene (Sigma) was added for HIV infected cell cultures. CEM clone 13 cells are derived from the human lymphoid cell line CEM (ATCC-CCL119) and express the T4 antigen

to a high level. Five days after infection with HIV-1_{BRU} or HIV-2_{ROD} isolates, about 80-90% of the cells produce viral particles and can be identified by a cytopathic effect corresponding to vacuolization of cells and appearance of small syncytia.

The HUT-78 cell line is another human T4 positive lymphoid cell line (Gadzudar et al., 1980) that is highly permissive for the replication of SIVmac₁₄₂ (Daniel et al., 1985). Peripheral blood lymphocytes from healthy blood donors were stimulated for three days with 0.2% (w/v) phytohemagglutinin fraction P (Difco, Detroit, USA) in RPMI-1640 medium supplemented with 10% fetal calf serum. Cells were then cultured in RPMI-1640 medium containing 10% (v/v) T cell growth factor (TCGF, Biotest). After infection with HIV-2, lymphocytes were cultured in presence of 10% (v/v) TCGF and 2 µg/ml Polybrene.

C. Metabolic Labeling of Cells

For metabolic labeling of proteins, infected cells were incubated for 16 hours at 37°C in MEM culture medium without L-methionine and serum but supplemented with 200 µCi/ml ³⁵S-methionine. For metabolic labeling of glycoproteins, infected cells were incubated for 16 hours at 37°C in MEM culture medium lacking serum and glucose but supplemented with 200 µCi/ml ³H-fucose or 200 µCi/ml ³H-glucosamine or 25 µCi/ml ¹⁴C-mannose.

D. Cell and Viral Extracts

Cell pellets corresponding to 10^7 cells were resuspended in 100 μ l of buffer: 10mM Tris-HCl pH 7.6, 150mM NaCl, 1mM EDTA, 0.2mM PMSF, 100 units/ml aprotinin (Iniprol, Choay) before addition of 100 μ l of the same buffer containing 2% (v/v) Triton

X-100. Cell extracts were centrifuged at 12,000 g for 10 minutes, and the supernatant was stored at -80°C until used. For viral extract preparations, 100 μ l of 10X lysis buffer (100mM Tris-HCl pH 7.6, 1.5M NaCl, 10mM EDTA, 10% (v/v) Triton X-100, 100 units/ml aprotinin) was added per ml of clarified supernatant from infected CEM cells and processed as above.

E. Preparation of an Immunoabsorbant with Antibodies from an HIV-2 Seropositive Patient Sera

Immunoglobulins from the serum of an HIV-2 seropositive patient were precipitated with 50% $(\text{NH}_4)_2\text{SO}_4$, dissolved in 20mM sodium phosphate (pH 8.0) and further purified on a DEAE cellulose column (DE 52, Whatman) by elution with 20mM sodium phosphate (pH 8.0). Immunoglobulins purified in this manner were judged to be 90% pure. The antibodies were subsequently coupled to CNBr-activated Sepharose CL 4B according to a technique described by Berg (1977). Two milligrams of IgG were coupled per ml of Sepharose CL 4B. This immunoabsorbant is referred to as HIV-2 serum-Sepharose.

F. Binding of the HIV-2 Proteins on the Immunoaffinity Column

Cell extracts from HIV-2 producing CEM cells were first diluted in two volumes of binding buffer (20mM Tris-HCl pH 7.6, 50mM KCl, 150mM NaCl, 1mM EDTA, 1% (v/v) Triton X-100, 20% (v/v) glycerol, 7mM mercaptoethanol, 0.2mM PMSF, 100 units/ml aprotinin) before incubation with one volume of HIV-2 serum-Sepharose. Supernatants from HIV-2 producing cells were processed as cell extracts except that only one tenth of binding buffer concentrate 10X was added per volume of supernatant. The binding was carried out overnight, then the column was washed batchwise in binding buffer. Proteins bound to the column were eluted by boiling in electrophoresis sample buffer (125mM Tris-HCl pH 6.8, 1% (w/v) SDS, 2M urea, 20% glycerol, 1% β -mercaptoethanol). Eluted proteins were resolved by electrophoresis on 7.5% polyacrylamide-SDS gels containing 6M urea and 0.1% bisacrylamide instead of 0.2% (w/v).

G. Preparative Electrophoresis

HIV-2 glycoproteins eluted from the affinity column were resolved by polyacrylamide gel electrophoresis as previously described, and the regions of the gel containing the viral glycoproteins were cut out by reference to the position of prestained molecular weight protein markers (BRL).

Glycoproteins were eluted by incubation for 16 hours at 4°C in elution buffer (0.1M NaHCO₃, 0.5mM EDTA, 0.05% (w/v) SDS, 0.2mM PMSF). The glycoprotein fractions thus obtained were lyophilized and kept refrigerated until used.

H. Two Dimensional Electrophoresis

Two dimensional gel electrophoresis was performed as described by O'Farrel (1975) with the following modification: L-(³⁵S)-methionine labeled proteins bound on the HIV-2 serum-Sepharose column were eluted by boiling in electrophoresis sample buffer as previously described before dilution in a volume of buffer containing 9.5M urea, 8% (v/v) mercaptoethanol, 1.6% (w/v) ampholines pH ranges 6.5-9, 0.4% (w/v) ampholines pH ranges 3-10, and 100 units/ml aprotinin.

It will be understood that the present invention is intended to encompass the previously described proteins and glycoproteins in purified form, whether or not fully glycosylated, and whether obtained using the techniques described herein or other methods. In a preferred embodiment of this invention, the polypeptides are substantially free of human tissue and human tissue components, nucleic acids, extraneous proteins and lipids, and adventitious microorganisms, such as bacteria and viruses. It will also be understood that the invention encompasses equivalent proteins and glycoproteins having substantially the same biological and immunogenic properties. Thus, this invention is intended to cover serotypic variants of the proteins and glycoproteins of the invention.

The proteins and glycoproteins of this invention can be obtained by culturing HIV-2 in susceptible mammalian cells of lymphocytic lineage, such as T-lymphocytes or pre-T-lymphocytes of human origin or non-human primate origin (e.g. chimpanzee, African green monkey, or macaques.) A number of different lymphocytes expressing the CD4 phenotypic marker can be employed. Examples of suitable target cells for HIV-2 infection are mononuclear cells prepared from peripheral blood, bone marrow, and other tissues from patients and donors. Alternatively, established cell lines can be employed. For example, HIV-2 can be propagated on blood-donor lymphocyte cultures, followed by propagation on continuous cell strains of leukemic origin, such as HUT 78. HUT 78 is a well characterized mature human T cell line, which has been deposited at Collection Nationale Des Cultures De Micro- organismes (C.N.C.M.) at the Institut Pasteur in Paris, France on February 6, 1986, under culture collection deposit accession number C.N.C.M. 1-519. Another suitable target for HIV-2 infection and production of the proteins and glycoproteins of the invention is the T-cell line derived from an adult with lymphoid leukemia and termed HT. HT cells continuously produce virus after parental cells are repeatedly exposed to concentrated cell culture fluids harvested from short-term culture T-cells grown in TCGF that originated from patients with LAS or AIDS. In addition, there are several other T or pre-T human cell lines, such as CEM and MOLT 3, that

can be infected and continue to produce HIV-2. Furthermore, B-lymphoblastic cell lines can also be productively infected by HIV. Montagnier et al., Science, 225:63-66 (1984).

The proteins and glycoproteins of the invention can be produced in the target cells using the culture conditions previously described, as well as other standard techniques. For instance, infected human lymphocytes can be stimulated for three days by phytohemagglutinin (PHA). The lymphocytes can be cultured in RPMI-1640 medium to which has been added 10% fetal calf serum, 10^{-5} M beta-mercaptoethanol, interleukin-2, and human alpha anti-interferon serum. Barre-Sinoussi et al., Science, 220:868-871 (1983). In addition, techniques for the propagation of HIV-2 in HUT 78 and CEM cell lines are described in U.S. application Serial No. 835,228, filed March 3, 1986, now U.S. Patent No. 4,839,288, the entire disclosure of which is relied upon and specifically incorporated by reference.

The production of virus in the cell cultures can be monitored using several different techniques. Supernatant fluids in the cell cultures can be monitored for viral reverse transcriptase activity. Electron microscopic observation of fixed and sectioned cells can also be used to detect virus. In addition, virus can be detected by transmitting the virus to fresh normal human T-lymphocytes (e.g., umbilical cord blood, adult peripheral blood, or bone marrow leukocytes) or to established T-cell lines. Testing for antigen expression by

indirect immunofluorescence or Western Blot procedures using serum from seropositive donors can also be employed. In addition, nucleic acid probes can be utilized to detect viral production.

After a sufficient period of time for viral multiplication to take place, infected cells can be separated from the culture medium and disrupted to expose intracellular proteins using conventional techniques. For example, physical shearing, homogenization, sonication, detergent solubilization, or freeze-thawing can be employed. The viral proteins released by these cells can be separated from the other cellular components and purified using standard biochemical procedures. For example, proteins can be separated from the live virus by centrifugation, and the proteins can then be purified by ultracentrifugation, gel filtration, ion-exchange chromatography, affinity chromatography, dialysis, or by the use of monoclonal antibodies or by combinations of these procedures. A thorough purification of the antigens of the invention can be performed by immunoreaction with the sera of patients known to possess antibodies effective against the antigens, with concentrated antibody preparations such as polyclonal antibodies, or with monoclonal antibodies directed against the antigens of the invention.

The proteins and the glycoproteins of the present invention can be used as antigens to identify antibodies to HIV-2 and SIV in materials and to determine the concentration of the antibodies in those materials. Thus, the antigens can be used for qualitative or quantitative determination of the retrovirus in a material. Such materials of course include human tissue and human cells, as well as biological fluids, such as human body fluids, including human sera. When used as a reagent in an immunoassay for determining the presence or concentration of the antibodies to HIV-2, the antigens of the present invention provide an assay that is convenient, rapid, sensitive, and specific.

More particularly, the antigens of the invention can be employed for the detection of HIV-2 by means of immunoassays that are well known for use in detecting or quantifying humoral components in fluids. Thus, antigen-antibody interactions can be directly observed or determined by secondary reactions, such as precipitation or agglutination. In addition, immunoelectrophoresis techniques can also be employed. For example, the classic combination of electrophoresis in agar followed by reaction with antiserum can be utilized, as well as two-dimensional electrophoresis, rocket electrophoresis, and immunolabeling of polyacrylamide gel patterns (Western Blot or immunoblot.) Other immunoassays in which the antigens of the present invention can be employed include, but are not limited to, radioimmunoassay, competitive immunoprecipitation assay,

enzyme immunoassay, and immunofluorescence assay. It will be understood that turbidimetric, colorimetric, and nephelometric techniques can be employed. An immunoassay based on Western Blot technique is preferred.

Immunoassays can be carried out by immobilizing one of the immunoreagents, either an antigen of the invention or the antibodies to the antigen, on a carrier surface while retaining immunoreactivity of the reagent. The reciprocal immunoreagent can be unlabeled or labeled in such a manner that immunoreactivity is also retained. These techniques are especially suitable for use in enzyme immunoassays, such as enzyme linked immunosorbent assay (ELISA) and competitive inhibition enzyme immunoassay (CIEIA).

When either the antigen of the invention or antibody to the antigen is attached to a solid support, the support is usually a glass or plastic material. Plastic materials molded in the form of plates, tubes, beads, or disks are preferred. Examples of suitable plastic materials are polystyrene and polyvinyl chloride. If the immunoreagent does not readily bind to the solid support, a carrier material can be interposed between the reagent and the support. Examples of suitable carrier materials are proteins, such as bovine serum albumin, or chemical reagents, such as glutaraldehyde or urea. Coating of the solid phase can be carried out using conventional techniques.

Depending on the use to be made of the proteins and glycoproteins of the invention, it may be desirable to label them. Examples of suitable labels are radioactive labels, enzymatic labels, fluorescent labels, chemiluminescent labels, and chromophores. The methods for labeling proteins and glycoproteins of the invention do not differ in essence from those widely used for labeling immunoglobulin. The need to label may be avoided by using labeled antibody to the antigen of the invention or anti-immunoglobulin to the antibodies to the antigen as an indirect marker.

Once the proteins and glycoproteins of the invention have been obtained, they can be used to produce polyclonal and monoclonal antibodies reactive therewith. Thus, a protein or glycoprotein of the invention can be used to immunize an animal host by techniques known in the art. Such techniques usually involve inoculation, but they may involve other modes of administration. A sufficient amount of the protein or the glycoprotein is administered to create an immunogenic response in the animal host. Any host that produces antibodies to the antigen of the invention can be used. Once the animal has been immunized and sufficient time has passed for it to begin producing antibodies to the antigen, polyclonal antibodies can be recovered. The general method comprises removing blood from the animal and separating the serum from the blood. The serum, which contains antibodies to the antigen, can be used as an antiserum to the antigen. Alternatively, the antibodies can be

recovered from the serum. Affinity purification is a preferred technique for recovering purified polyclonal antibodies to the antigen from the serum.

Monoclonal antibodies to the antigens of the invention can also be prepared. One method for producing monoclonal antibodies reactive with the antigens comprises the steps of immunizing a host with the antigen; recovering antibody-producing cells from the spleen of the host; fusing the antibody-producing cells with myeloma cells deficient in the enzyme hypoxanthine-guanine phosphoribosyl transferase to form hybridomas; selecting at least one of the hybridomas by growth in a medium comprising hypoxanthine, aminopterin, and thymidine; identifying at least one of the hybridomas that produces an antibody to the antigen; culturing the identified hybridoma to produce antibody in a recoverable quantity; and recovering the antibodies produced by the cultured hybridoma.

These polyclonal or monoclonal antibodies can be used in a variety of applications. Among these is the neutralization of corresponding proteins. They can also be used to detect viral antigens in biological preparations or in purifying corresponding proteins, glycoproteins, or mixtures thereof, for example when used in affinity chromatographic columns.

The invention provides immunogenic proteins and glycoproteins, and more particularly, protective polypeptides for use in the preparation of vaccine compositions against HIV-2. These polypeptides can thus be employed as viral vaccines

by administering the polypeptides to a mammal susceptible to HIV-2 infection. Conventional modes of administration can be employed. For example, administration can be carried out by oral, respiratory, or parenteral routes. Intradermal, subcutaneous, and intramuscular routes of administration are preferred when the vaccine is administered parenterally.

The ability of the proteins, glycoproteins, and vaccines of the invention to induce protective levels of neutralizing antibody in a host can be enhanced by emulsification with an adjuvant, incorporation in a liposome, coupling to a suitable carrier, or by combinations of these techniques. For example, the proteins and glycoproteins of the invention can be administered with a conventional adjuvant, such as aluminum phosphate and aluminum hydroxide gel, in an amount sufficient to potentiate humoral or cell-mediated immune response in the host. Similarly, the polypeptides can be bound to lipid membranes or incorporated in lipid membranes to form liposomes. The use of nonpyrogenic lipids free of nucleic acids and other extraneous matter can be employed for this purpose.

The immunization schedule will depend upon several factors, such as the susceptibility of the host to infection and the age of the host. A single dose of the vaccine of the invention can be administered to the host or a primary course of immunization can be followed in which several doses at intervals of time are administered. Subsequent doses used as boosters can be administered as needed following the primary course.

The proteins and vaccines of the invention can be administered to the host in an amount sufficient to prevent or inhibit HIV-2 infection or replication *in vivo*. In any event, the amount administered should be at least sufficient to protect the host against substantial immunosuppression, even though HIV infection may not be entirely prevented. An immunogenic response can be obtained by administering the proteins or glycoproteins of the invention to the host in an amount of about 10 to about 500 micrograms antigen per kilogram of body weight, preferably about 50 to about 100 micrograms antigen per kilogram of body weight. The proteins and vaccines of the invention can be administered together with a physiologically acceptable carrier. For example, a diluent, such as water or a saline solution, can be employed.

In summary, proteins and glycoproteins, which are precursors of HIV-2 and SIV envelope protein, have now been identified. In addition to providing useful tools for detection of antibodies to the retrovirus in humans and for raising neutralizing antibodies to HIV-2 *in vitro* and *in vivo*, this invention adds to the base of knowledge relating to immunodeficiency active proteins and glycoproteins of the AIDS

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viruses. The reference molecular weight was in the form of the following dye markers marketed by BRL Co.:

myosine 200 Kd
phosphorylase B 92.7 Kd
BSA 68 Kd
ovalbumin 43 Kd
alpha chymotrypsin 25.7 Kd
beta lactoglobulin 18.4 Kd
lysozyme 14.3 Kd.

Molecular weights were estimated within an accuracy of about $\pm 10\%$.

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WHAT IS CLAIMED IS:

1. An isolated immune complex comprising a protein and an antibody that binds with said protein, wherein the protein is selected from the group consisting of gp300 of HIV-2, p200 of HIV-2, p90/80 of HIV-2, and gp300_{SIV}.
2. The immune complex of claim 1, wherein the antibody, protein, or both the antibody and protein, are labeled with an immunoassay label selected from the group consisting of radioisotopes, enzymes, fluorescent labels, chemiluminescent labels, and chromophore labels.
3. An isolated antibody which binds with a protein selected from the group consisting of gp300 of HIV-2, p200 of HIV-2, p90/80 of HIV-2, and gp300_{SIV}.
4. The antibody of claim 3, wherein the antibody is labeled with an immunoassay label selected from the group consisting of radioisotopes, enzymes, fluorescent labels, chemiluminescent labels, and chromophore labels.
5. An immunogenic composition comprising a pharmaceutically effective amount of one or more proteins of human immunodeficiency virus type 2 (HIV-2) and a pharmaceutically acceptable carrier, wherein said proteins are selected from the group consisting of gp300, p200, and p90/80 of HIV-2.

6. An in vitro diagnostic method for detecting infection of cells by human immunodeficiency virus type 2 (HIV-2), comprising:

- a) providing a composition comprising cells suspected of being infected with HIV-2;
- b) disrupting cells in the composition to expose intracellular proteins; and
- c) assaying the exposed intracellular proteins for the presence of one or more proteins selected from the group consisting of gp300 of HIV-2, p200 of HIV-2, p90/80 of HIV-2, and gp300_{SIV},

wherein the presence of said one or more proteins is indicative of the presence of HIV-2.

7. The method of claim 6, wherein the assaying of exposed intracellular proteins is carried out by a method selected from the group consisting of electrophoresis of the proteins and immunoassay of the proteins with antibodies that are immunologically reactive with gp300 of HIV-2, p200 of HIV-2, p90/80 of HIV-2, or gp300_{SIV}.

8. The method of claim 7, wherein the antibodies are labeled with an immunoassay label selected from the group consisting of radioisotopes, enzymes, fluorescent labels, chemiluminescent labels, and chromophore labels.

9. An in vitro method for detecting antigens of human immunodeficiency virus type 2 (HIV-2), comprising:

- a) providing a composition suspected of containing antigens of HIV-2; and
- b) assaying the composition for the presence of one or more proteins selected from the group consisting of gp300, p200, and p90/80 of HIV-2,

wherein the presence of said one or more proteins is indicative of the presence of antigens of HIV-2.

10. The method of claim 9, wherein said assaying of the composition is carried out by a method selected from the group consisting of electrophoresis of said proteins and immunoassay with antibodies that are immunologically reactive with gp300, p200, or p90/80 of HIV-2.

11. The method of claim 10, wherein the antibodies are labeled with an immunoassay label selected from the group consisting of radioisotopes, enzymes, fluorescent labels, chemiluminescent labels, and chromophore labels.

12. An in vitro diagnostic method of distinguishing HIV-2 infection, or co-infection of HIV-1 and HIV-2, from HIV-1 infection in cells comprising:

- a) providing an extract comprising intracellular proteins of said cells; and

- b) assaying said extract for the presence of one or more proteins selected from the group consisting of gp300 of HIV-2, p200 of HIV-2, p90/80 of HIV-2, and gp300_{SIV},

wherein the presence of said one or more proteins is indicative of the presence of HIV-2 infection or co-infection of HIV-1 and HIV-2.

13. The method of claim 12, wherein said assaying of the extract is carried out by a method selected from the group consisting of electrophoresis of said proteins and immunoassay with antibodies that are immunologically reactive with gp300, p200, or p90/80 of HIV-2.

14. The method of claim 13, wherein the antibodies are labeled with an immunoassay label selected from the group consisting of radioisotopes, enzymes, fluorescent labels, chemiluminescent labels, and chromophore labels.

15. An *in vitro* diagnostic method for detecting the presence or absence of antibodies which bind to a protein of HIV-2, comprising:

- a) contacting one or more proteins of HIV-2 selected from the group consisting of p90/80, p200, and gp300 of HIV-2 with a biological fluid for a time and under conditions sufficient for said proteins and antibodies in the biological fluid to form a protein-antibody immune complex; and

b) detecting the formation of the complex.

16. The method of claim 15, wherein the detecting step further comprises measuring the formation of said immune complex.

17. The method of claim 15, wherein said one or more proteins are labeled with an immunoassay label selected from the group consisting of radioisotopes, enzymes, fluorescent labels, chemiluminescent labels, and chromophore labels.

18. An *in vitro* diagnostic kit for detecting the presence or absence of antibodies which bind to a protein of HIV-2, comprising:

- a) one or more proteins of HIV-2 selected from the group consisting of p90/80, p200, and gp300 of HIV-2; and
- b) means for detecting the formation of immune complex between said proteins and said antibodies;

wherein the proteins and the means are present in an amount sufficient to perform said detection.

19. The kit of claim 18, wherein the means for detecting the formation of the immune complex is an assay selected from the group consisting of radioimmunoassay, immunoenzymatic assay, and immunofluorescent assay.

20. An in vitro method for detecting antibodies in a sample of human body fluid which specifically bind to antigenic sites of an antigen, comprising:

- a) contacting said antigen with antibodies from human body fluid for a time and under conditions sufficient to permit formation of an antigen-antibody complex between said antigen and said antibodies; and
- b) detecting the formation of said antigen-antibody complex,

wherein said antigen comprises a protein selected from the group consisting of p90/80 of HIV-2, p200 of HIV-2, gp300 of HIV-2, and gp300_{SIV}.

ABSTRACT

Four glycoproteins of apparent molecular weights 300,000, 140,000, 125,000, and 36,000 (gp300, gp140, gp125, and gp36) are detectable in human immunodeficiency virus type 2 (HIV-2) infected cells. The gp125 and gp36 are the external and transmembrane components, respectively, of the envelope glycoproteins of HIV-2 mature virions. The gp300, which is a dimeric form of gp140, the precursor of HIV-2 envelope glycoprotein, is probably formed by a pH dependent fusion in the endoplasmic reticulum. Such a doublet is also observed in cells infected with simian immunodeficiency virus (SIV), a virus closely related to HIV-2. On the other hand, the envelope glycoprotein precursor of HIV-1 does not form a dimer during its processing. Experiments carried out with various inhibitors of oligosaccharide trimming enzymes suggest that transient dimerization of the glycoprotein precursor is required for its efficient transport to the Golgi apparatus and for its processing. The gp300 is useful for detecting antibodies to HIV-2 antigens in human body fluids and for raising antibodies to gp300.



FIG. 1A

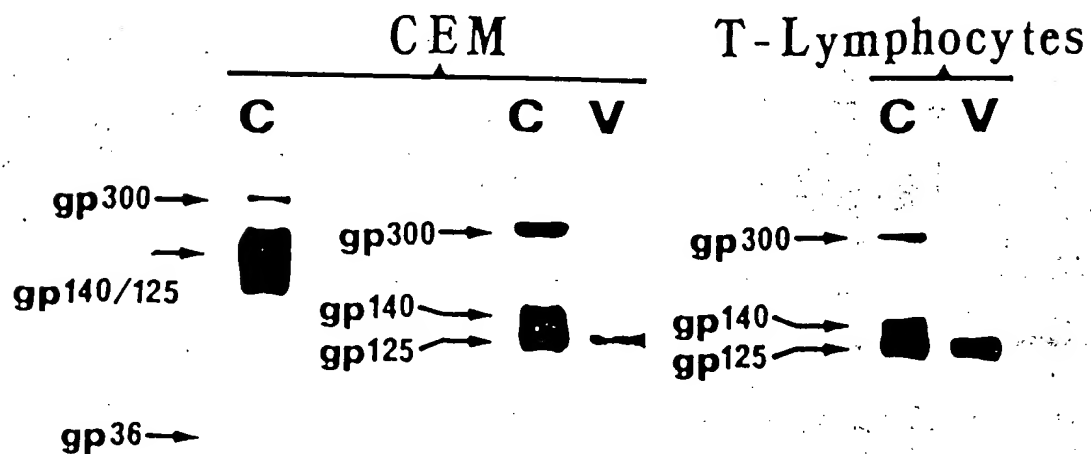


FIG. 1B

FIG. 2

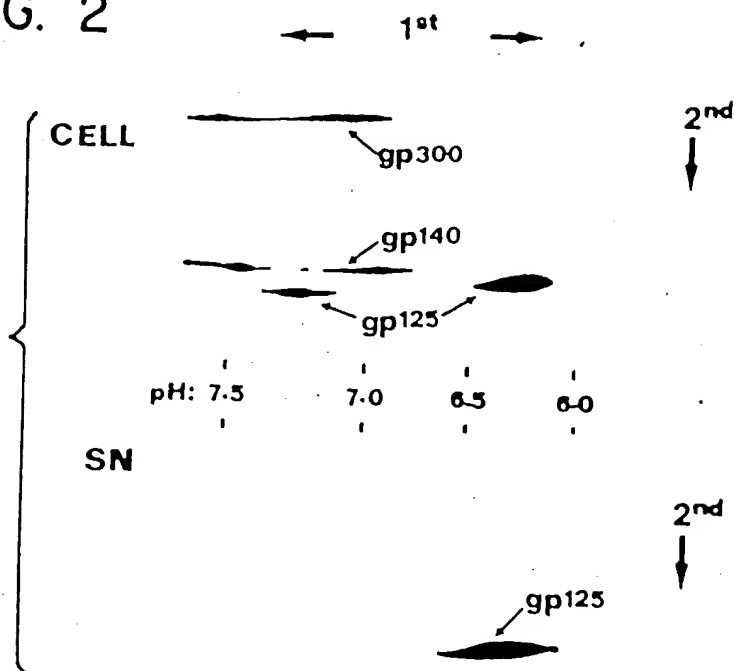


FIG. 3A

FIG. 3B

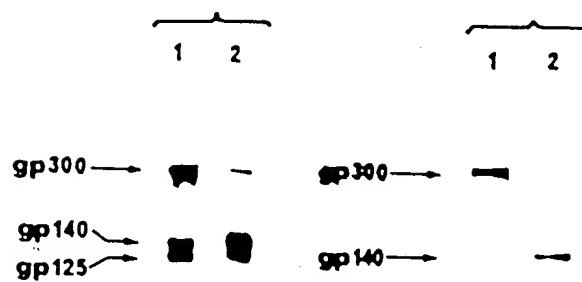
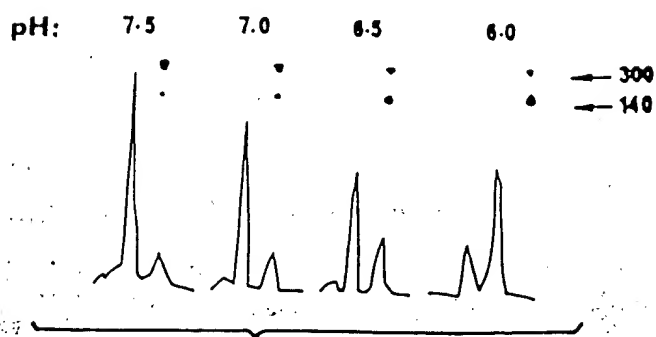


FIG. 3C



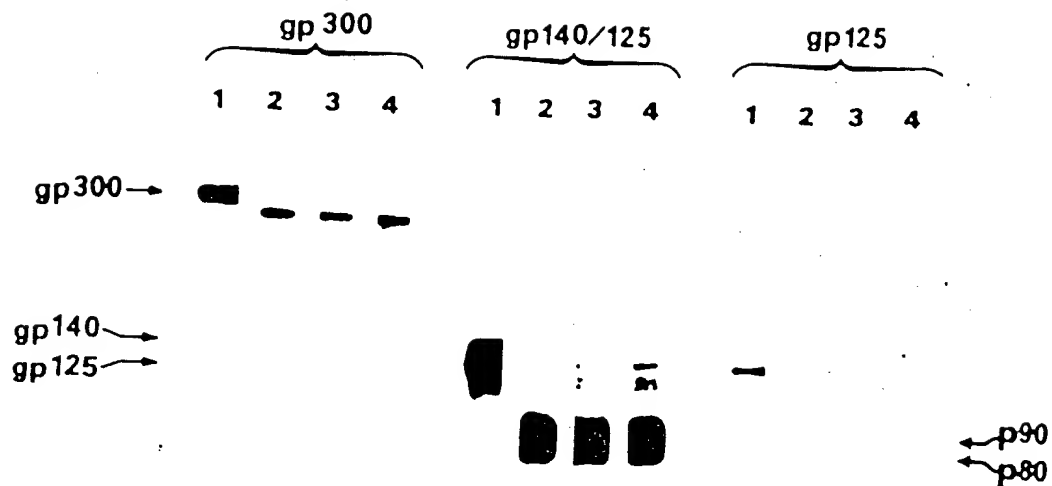


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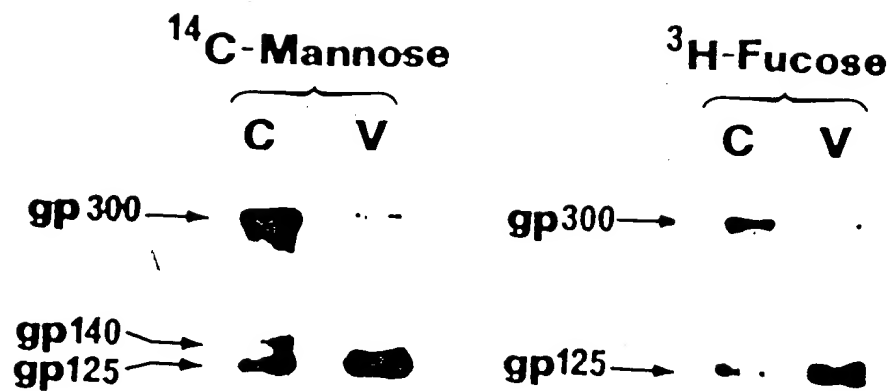


FIG. 5

FIG. 6

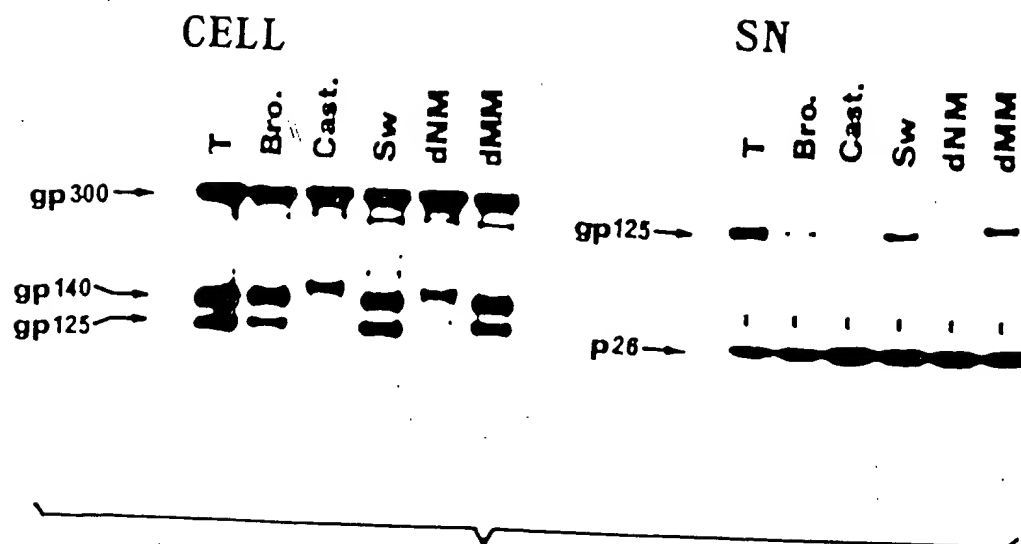
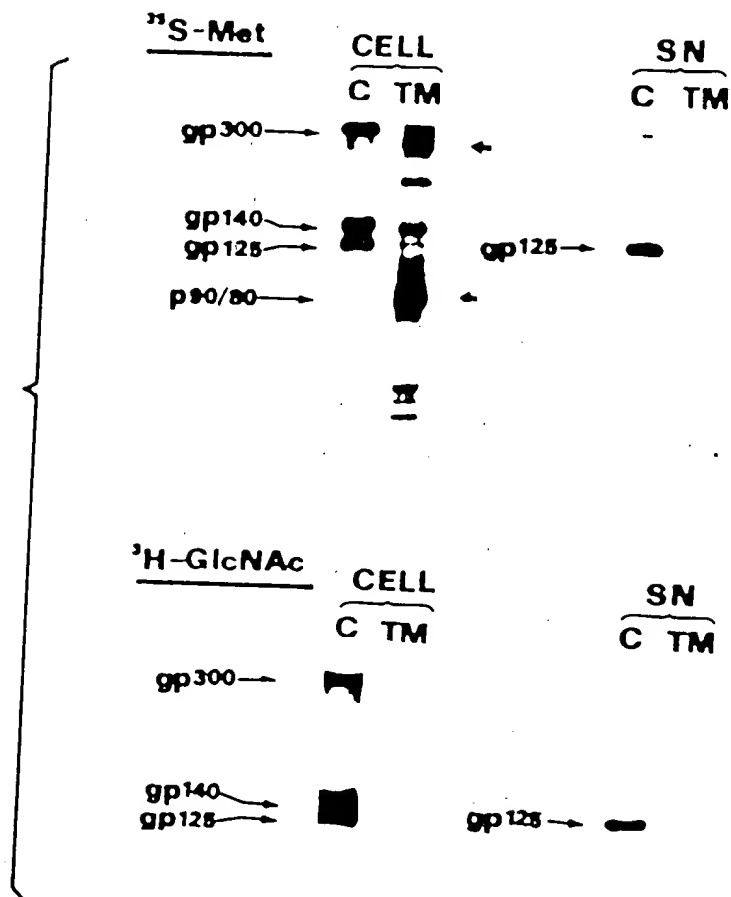


FIG. 7

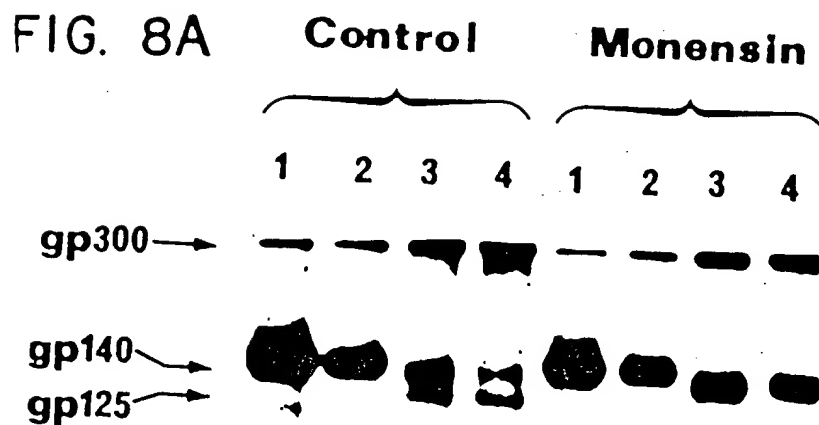
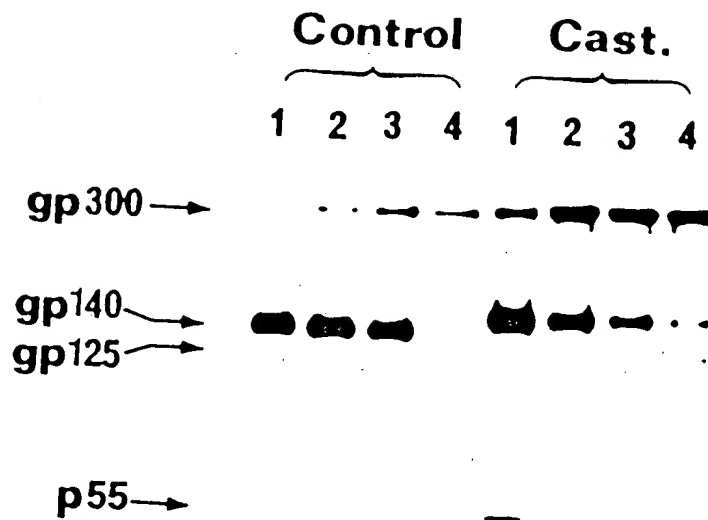


FIG. 8B

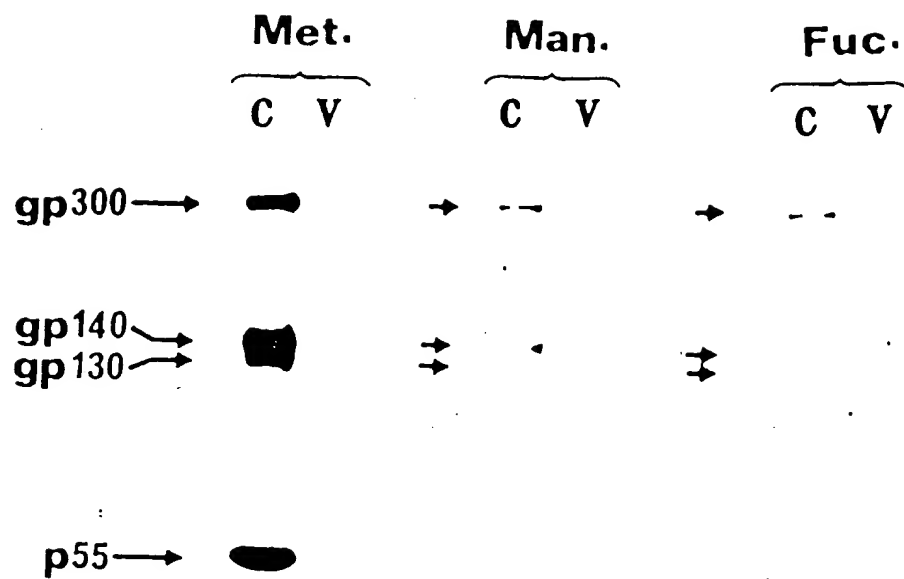


FIG. 9

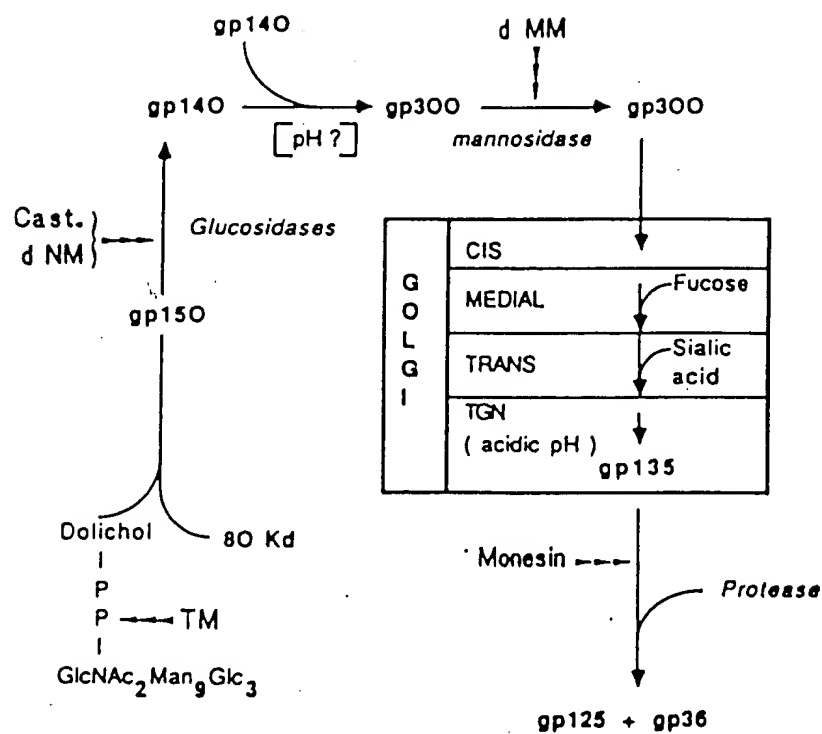


FIG. 10

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Monoclonal Antibodies Recognizing a Neoantigen of Poly(C9) Detect the Human Terminal Complement Complex in Tissue and Plasma

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Mollnes, T.E., Lea, T., Harboe, M. & Tschopp, J. Monoclonal Antibodies Recognizing a Neoantigen of Poly(C9) Detect the Human Terminal Complement Complex in Tissue and Plasma. *Scand. J. Immunol.* 22, 183-195, 1985.

The terminal complement complex (TCC), consisting of C5b, C6, C7, C8, and C9, contains neoantigens that are absent from the individual native components. Neoantigens are present both in the membrane-bound (MAC) and the fluid-phase (SC5b-9) complex. The present study describes production of monoclonal antibodies against neoantigens of both forms of the TCC. A convenient screening and detection system, based mainly on enzyme-linked immunosorbent assays, crossed immunoelectrophoresis with autoradiography, and affinity chromatography with subsequent sodium dodecyl sulphate-polyacrylamide gel electrophoresis including immunoblotting, is described in detail. Two monoclonal antibodies were specific for a neoantigen located in the poly(C9) moiety of the TCC. One of these antibodies, MCaE11, was used for immunohistochemical detection of MAC in tissue and for quantification of the fluid-phase TCC in ethylenediaminetetraacetic acid plasma.

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The terminal complement complex (TCC) consists of the components C5b, C6, C7, C8, and C9. It exists in two analogous forms: the membrane attack complex (MAC) [10-12], which is the mediator of complement lysis, and the fluid-phase non-lytic SC5b-9 complex [20, 22], which in addition to the terminal components contains the S-protein [21] of human plasma.

The MAC has been demonstrated both in vitro and in vivo, whereas the SC5b-9 complex is characterized only in vitro. Recently, however, a TCC has been detected in normal human plasma by means of an assay based on antibodies against the native components [17]. This assay requires separation of the native components from the complex and thus cannot be used for quantification of large series of plasma samples.

Neoantigens [13] are new antigenic determi-

nants that appear in the TCC upon its assembly, whereas they cannot be detected in the individual native components. Several xenoantiseria [1, 4] and one monoclonal antibody [7] against neoantigens have been described. These antibodies have been used to examine the MAC in tissue, but they could not be used for detection of the TCC in normal human plasma.

The aims of the present study were to produce monoclonal antibodies highly specific for neoantigens of the TCC and to develop and standardize a convenient screening and detection system for these antibodies. We obtained two monoclonal antibodies, MCaE11 and MCbC5, which were specific for a neoantigen of poly(C9). The MCaE11 antibody was used for immune fluorescence to detect the MAC in tissue and for quantification of the fluid-phase TCC in plasma samples by an enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Specifications of materials

Tween-20, Triton X-100, zymosan A, and diaminobenzidine were purchased from Sigma Chemical Co., St Louis, Mo., USA; Zwittergent 3-14, anti-C6 (lot 405673), and anti-C7 (lot 201756) from Calbiochem-Behring Corp., San Diego, Calif., USA; anti-C5 (lot 031B), anti-IgM (lot 037), and anti-human serum (lot 013A) from Dakopatts, Copenhagen, Denmark; anti-C8 (lot IgG-009-2) from Atlantic Antibodies, Scarborough, Me., USA; and anti-C3c (lot 153311B), anti-C4 (lot 153512E), anti-C9 (lot 104303E), and anti-goat Ig peroxidase (lot 141206A) from Behringwerke AG, Marburg, FRG. Peroxidase-conjugated anti-mouse Ig (lot 9) and anti-rabbit Ig (lot 6) were obtained from Amersham International, UK. A monoclonal anti-IgM (IIIG2) antibody was produced in our own laboratory. Normal mouse IgG was isolated on a protein A column as described [9]. A polyclonal antiserum against neoantigens of the MAC was raised in rabbits and absorbed as described [4].

Nunc Immunoplate II (Copenhagen, Denmark) was used in the ELISA experiments. Coating buffer was phosphate-buffered saline (PBS) containing 0.02% NaN_3 . Incubation and washing buffer was PBS containing 0.1% Tween 20. The plates were washed in a Dynawasher three times between each incubation, and optical density was read on a Dynatec MR580 at 405 nm after 20–30 min. Coating was performed at 4°C for at least 48 h, whereas all other incubations were made at 37°C for 45 min. Substrate was 2,2'-azino-di-(3-ethylbenzthiazoline sulphonic acid) (ABTS) from Boehringer Mannheim, FRG. ABTS was diluted in 0.1 M sodium acetate, pH 4.0 (180 mg ABTS/l), and 3% H_2O_2 was added to a final concentration of 0.8 $\mu\text{g}/\text{ml}$ immediately before use.

Sephacryl S-300, Sephacryl S-400, Sepharose 4B, and Sepharose 6B were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Gel filtrations of zymosan-activated serum and normal ethylenediaminetetraacetic acid (EDTA) plasma were performed in a 0.05 M Tris/HCl buffer, pH 7.60, containing 10 mM EDTA and 0.01% Triton X-100. A 0.005 M sodium borate buffer, pH 8.8, containing 0.05 M sodium chloride, 0.02% NaN_3 , and 0.02% Zwittergent 3-14, was used for gel filtration of the membrane extract.

Pools of normal human EDTA plasmas and normal human sera from healthy blood donors were kindly supplied by the Red Cross Blood Centre, Oslo. They were stored at -70°C . Zymosan activation was done by adding 10 mg zymosan/ml serum, which was incubated with continuous mixing at 37°C for 1 h and spun in a Sorvall RC5 at 43,000 g for 45 min.

Immunization

The MAC was produced and purified as described elsewhere [5, 30]. In brief, washed rabbit erythrocytes were lysed by normal human serum containing [^{125}I]C6. The pellet was washed and treated with sodium borate buffer containing 1% Zwittergent 3-14

for protein extraction. The supernatant was passed through a Sephacryl S-400 column (2.5×90 cm) at 32 ml/h, and fractions containing MAC were pooled, concentrated, and used for immunization. Ten to 50 μg protein were injected into BALB/c mice, 50% subcutaneously and 50% intraperitoneally, together with Freund's complete adjuvant. The same amount of protein was injected subcutaneously after 2 weeks and, in some mice, after another 2 weeks. Three to 4 days before fusion, the same amount of protein in saline was injected intravenously.

Production of monoclonal antibodies

The monoclonal (MC) antibodies MCaE11 and MCbC5 were developed by fusing the myeloma cell line X63-Ag 8.653 and spleen cells from immunized mice. The procedure for fusion and selection of clones was mainly as described elsewhere [8]. In brief, spleen cells were mixed with myeloma cells in a ratio of 4:1 in the presence of 50% polyethylene glycol (PEG) 4000 (lot 2190553, Merck, Darmstadt, FRG). The cell suspension was seeded in flat-bottom 96-well plates (Nunc Delta, Nunc) in RPMI 1640 medium (Gibco, U.K.) with 20% fetal calf serum. The next day and the following 2 days, half of the medium was replaced by medium containing hypoxanthine supplemented with aminopterin and thymidine (HAT). Positive wells were scored by the ELISA method described below.

Ascitic fluid was produced by pretreating BALB/c mice with 0.5 ml pristane intraperitoneally and injecting 2×10^6 cells in 0.5 ml RPMI 1640 10 days later. The IgG fraction was isolated on a protein A column as described [9]. Isotypes of the monoclonal antibodies were determined by double immunodiffusion with class- and subclass-specific xenoantisera (Nordic Immunological Laboratories, Tilburg, The Netherlands).

Screening and detection system for the monoclonal antibodies

Screening procedure. ELISA plates coated with immunizing agent (1:1000), zymosan-activated human serum (1:10,000), and normal human EDTA plasma (1:10,000) were used for screening of culture supernatants, which were diluted 1:3 and tested with peroxidase-conjugated anti-mouse Ig (1:1000). Clones that were positive against immunizing agent and showed more than three times higher activity against activated serum than against plasma were selected and further cultured. Supernatants from these expanded clones were tested as described above and, in addition, against zymosan-treated EDTA plasma (1:10,000) and the void volume fraction (Sephacryl S-300) of activated serum (1:1000). Control antisera were used in the following dilutions: anti-C5 (1:2000), anti-C9 (1:2000), anti-C3c (1:50,000), anti-C4 (1:25,000), MC anti-IgM (1:1500), xenoantiserum against neoantigens (1:10,000), and the peroxidase-conjugated anti-rabbit Ig (1:1000).

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Gel filtration experiments. Gel filtration of 2.0 ml zymosan-activated serum and normal EDTA-plasma was performed both on a Sephacryl S-300 and on Sepharose 6B columns (1.5×90 cm) at 12 ml/h, and 4-ml fractions were collected. The detergent extract containing the MAC was passed through a Sephacryl S-400 column (2.5×90 cm) at 25 ml/h, and 8-ml fractions were collected. All fractions were diluted 1:50 in PBS (0.02% NaN₃) and coated onto ELISA plates. They were tested with the selected monoclonal antibodies and the same control antisera as described above for the screening.

Crossed immunoelectrophoresis. Radiolabelling of proteins with ¹²⁵I was done by the Iodogen method as described in Pierce Bio-Research Products Technical Bulletin (Pierce Chemical Co., Rockford, Ill., USA). Crossed immunoelectrophoresis (CIE) was performed with 1% agarose (human serum albumin (HSA), lot 3271, Litex, Denmark) in 0.02 M Tris/barbiturate buffer, pH 8.6. The first dimension was run at 8 V/cm for 1½ h and the second dimension at 3 V/cm for 18 h. One hundred microlitres of the xenoantisera were used in the top gels, and labelled monoclonal antibodies were added to a final concentration of 100 ng/ml. EDTA plasma and activated serum were applied undiluted in the wells. The plates were washed in PBS and stained with Coomassie brilliant blue R250. Autoradiographs were exposed for 1–3 days.

MCaE11 immunosorbent. Six milligrams IgG of MCaE11 were coupled to 3 ml Sepharose 4B by cyanogen bromide as described [15]. Normal plasma and activated serum were passed through the column. Effluent buffer was PBS, and elution buffer was 0.1 M glycine/HCl, pH 2.8, containing 0.15 M sodium chloride. The eluates were immediately dialysed against PBS. The effluents and eluates were then tested in a double-antibody ELISA described in detail elsewhere [17]. This ELISA detects the TCC by a combination of anti-C6 and anti-C5 and is independent of anti-neoantigen antibodies. The eluate was also examined in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), performed mainly as described elsewhere [14], and tested against anti-C5, anti-C6, anti-C7, anti-C8, anti-C9, MCaE11, and MCbC5 after electroblotting onto nitrocellulose.

Identification of the subunit within the MAC interacting with the monoclonal antibodies. The interaction of the monoclonal antibodies with the different subunits of the MAC was studied by western blot and immunodot blot analysis. For the western blots, 2 µg of purified C5b-6, C7, C8, and C9 [28] and poly(C9) [29] were subjected to SDS-PAGE [14]. A 2.5–10% gradient gel was used. Proteins were subsequently electroblotted onto nitrocellulose (Schleicher and Schüll) for 4 h at 15 V [27]. MCaE11 and MCbC5 were diluted to 0.1 µg/ml in 2% gelatin containing Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl) and incubated with the nitrocellulose sheet for 2 h. Bound antibodies were detected by means of anti-mouse IgG antibodies coupled to horseradish peroxidase (Sigma). The substrate was 4-chloro-1-naphthol.

Immunodot blot analysis was carried out using the Bio-dot apparatus (Bio-Rad, Richmond, Calif., USA). Fifty nanograms of purified C5, C6, C7, C8, C9, and poly(C9) were serially twofold diluted and adsorbed onto nitrocellulose sheets. Free sites were blocked by gelatin containing TBS and further processed exactly as described for the western blot technique.

Immunohistochemistry and fluorescence microscopy

Monospecific rabbit anti-mouse Ig was conjugated with fluorescein isothiocyanate (FITC) as described [6]. The FITC to protein (F/P) ratio was 2.7. Ascitic fluid (MCaE11) and control ascitic fluid (X63 Ag 8.653) were diluted 1:500. The microscope was a Leitz Ortholux with an XBO 150-W lamp, a Ploem illuminator, and an oil immersion objective (63×1.3 NA).

ELISA for detection of fluid-phase TCC

The monoclonal antibodies were tested in a double-antibody ELISA, based on the sandwich principle with one antibody coated onto the plate, then addition of the antigen, and finally another antibody was used in the second antibody layer to detect the antigen. First, plates were coated with anti-C6 (2 µg/ml), and 10-fold dilutions of the void-volume fractions (Sephacryl S-300) of activated serum and normal plasma were added. MCaE11 (0.2 µg/ml) and MCbC5 (0.4 µg/ml) were used in the second antibody step. MC anti-IgM in equal concentration was used as control. Second, the plates were coated with monoclonal antibodies (aE11, bC5, anti-IgM clone II G2) and normal mouse IgG in equal concentrations (1 µg/ml). Tenfold dilutions of unfractionated normal plasma and activated serum were tested and were detected by anti-C5 and anti-C9 in the second antibody layer.

The quantitative assay, based on these specificity control experiments, was performed as described in detail in the accompanying paper [16].

RESULTS

Monoclonal antibodies were produced and screened for their reactivity against neoantigens of the assembled TCC. At each step of the detection system we selected antibodies that showed patterns compatible with the pattern expected for antibodies directed against neoantigens of the TCC, whereas all the other clones were excluded. Finally, we ended up with two monoclonal antibodies, MCaE11 and MCbC5, specific for neoantigen of the TCC

Detection of anti-TCC neoantigen antibodies

Screening. The initial screening was based on activity against the immunizing agent. In addi-

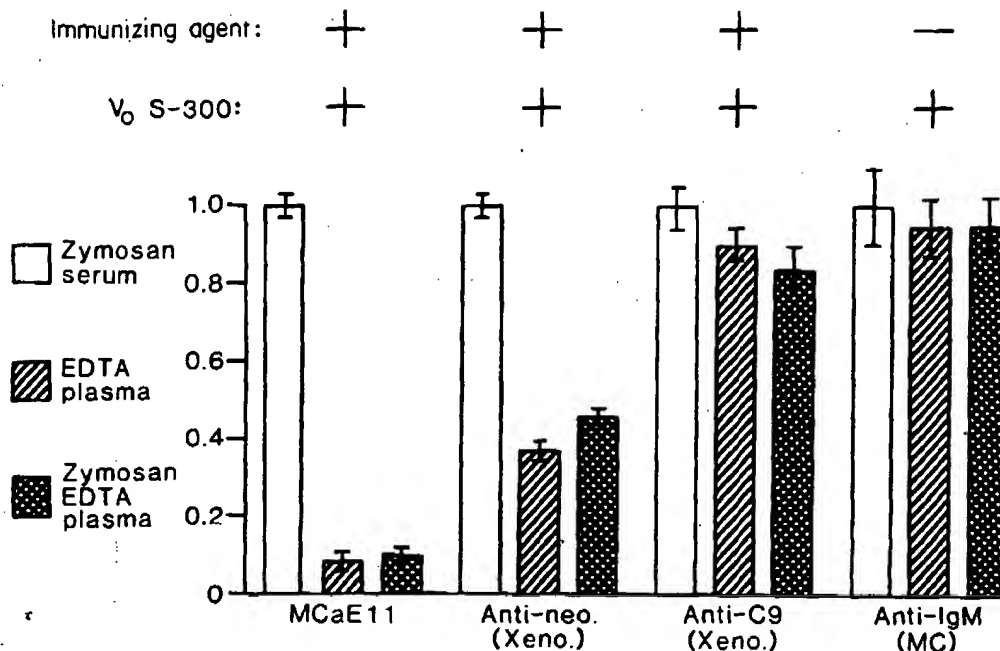


FIG. 1. Screening. Relative activity of different antibodies against five different antigens as determined by ELISA: immunizing agent (purified MAC); V₀ S-300 (void-volume fraction of zymosan-activated serum, mol. wt ≥ 700 kD); zymosan-activated serum; EDTA plasma; and zymosan-treated EDTA plasma. The activity against the immunizing agent and V₀ of S-300 are shown as positive or negative reactions. The results indicated by columns \pm SD can be compared directly because we used equal concentrations of serum and plasma, respectively, and the same ELISA plate for these three antigens. The optical density obtained for zymosan-activated serum was arbitrarily set to 1.0 for each experiment. The other values were referred to this relative optical density. Xeno.=xenoantiserum; Anti-neo.=anti-neoantigen.

tion, we looked for a marked difference in activity against normal human plasma and zymosan-activated serum. More than 95% of the 500 clones tested were positive against the immunizing agent, whereas less than 5% showed a difference in reactivity against plasma and activated serum. The few clones with a strong reaction against activated serum and a weak reaction against normal plasma were selected for further characterization. They were then tested against: (1) immunizing agent (MAC, positive control), (2) the void-volume fraction after gel filtration of zymosan-activated serum through a Sephacryl S-300 column (SC5b-9, positive control), (3) zymosan-activated whole human serum (positive control compared with plasma), (4) normal human EDTA plasma (negative control compared with activated serum), and (5) zymosan-treated EDTA plasma (negative control for unspecific zymosan effects).

We included the following three groups of antibody controls in these experiments: xenoantiserum against neoantigens, antibodies against

the native components in the complex, and antibodies against other plasma proteins. Some of these data are presented in Fig. 1. MCAE11 and MCbC5 showed high activity against activated serum compared with non-activated plasma. An absorbed xenoantiserum against neoantigens showed higher activity against normal plasma than the MCAE11 and the MCbC5. Antibodies against native terminal components (anti-C5 and anti-C9) and against other serum proteins (anti-C3c, anti-C4, and anti-IgM) showed no significant difference between activated serum and non-activated plasma. The results obtained with anti-C9 and monoclonal anti-IgM are illustrated in Fig. 1. All the antibodies shown in the figure showed positive reactions against the Sephacryl S-300 void-volume fraction of activated serum, and all except anti-IgM were positive against the immunizing agent, as expected.

Gel filtration of plasma and activated serum. Clones selected from the screening were then tested against individual fractions obtained after gel filtration of zymosan-activated serum and

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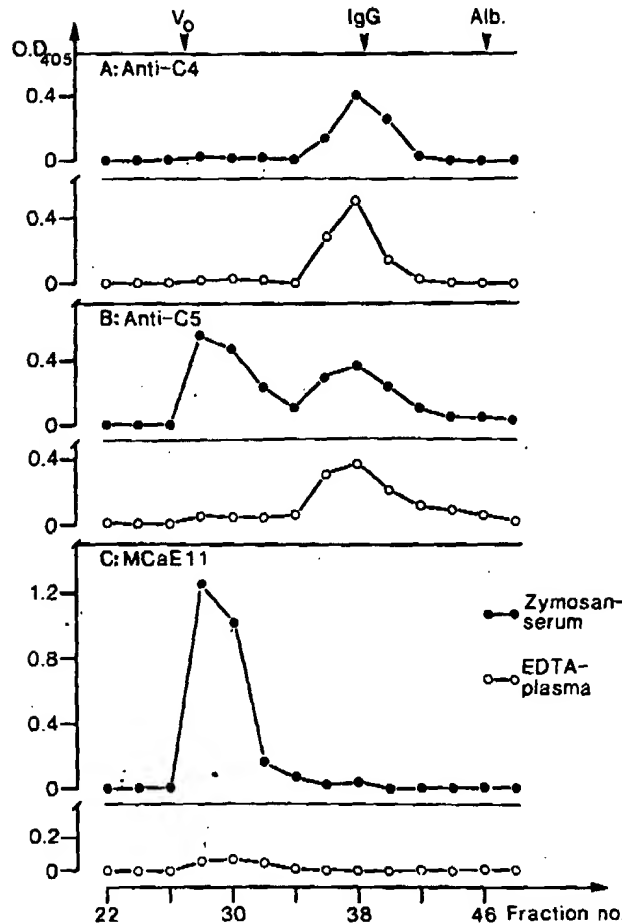


Fig. 2. Gel filtration experiments (SC5b-9). Individual fractions, obtained after passing zymosan-activated serum and normal EDTA plasma through a Sephacryl S-300 column, were coated onto ELISA plates and tested against anti-C4 (panel A), anti-C5 (panel B), and MCAE11 (panel C). The positions of V_0 (void-volume fraction), IgG, and albumin (Alb.) are indicated with arrowheads.

non-activated plasma. We used the same antibody controls as described for the screening. Some of these data are illustrated in Fig. 2. Panel A shows the reaction patterns with anti-C4, which were similar in zymosan-activated serum and in plasma. Anti-C5 (panel B) showed one peak in plasma, corresponding to native C5, and an additional peak in activated serum, corresponding to the TCC [17]. In panel C is shown the activity of MCAE11 against activated serum compared with a low but significant activity against plasma. The peak appeared in the void-volume fraction of Sephacryl S-300 and thus corresponds to a mol. wt of 700 kD or higher. A Sepharose 6B column with an exclusion limit of 4000 kD was used to examine the molecular

weight more exactly. The MCAE11 peak then corresponded to the IgM peak and to the first anti-C5 and anti-C9 peaks seen when activated serum was passed through the column and is therefore consistent with activity against the SC5b-9 complex of approximately 1000 kD [11].

Gel filtration of complement-lysed membrane extract. Detergent extract from complement-lysed rabbit erythrocytes was passed through a Sephacryl S-400 column (exclusion limit of approximately 8000 kD) (Fig. 3). The MAC was identified by antibodies against the native components in the complex, and a relatively heterogeneous elution peak was found, as described earlier [5, 19, 26]. MCAE11 showed exactly the same pattern. The position of IgM is indicated in

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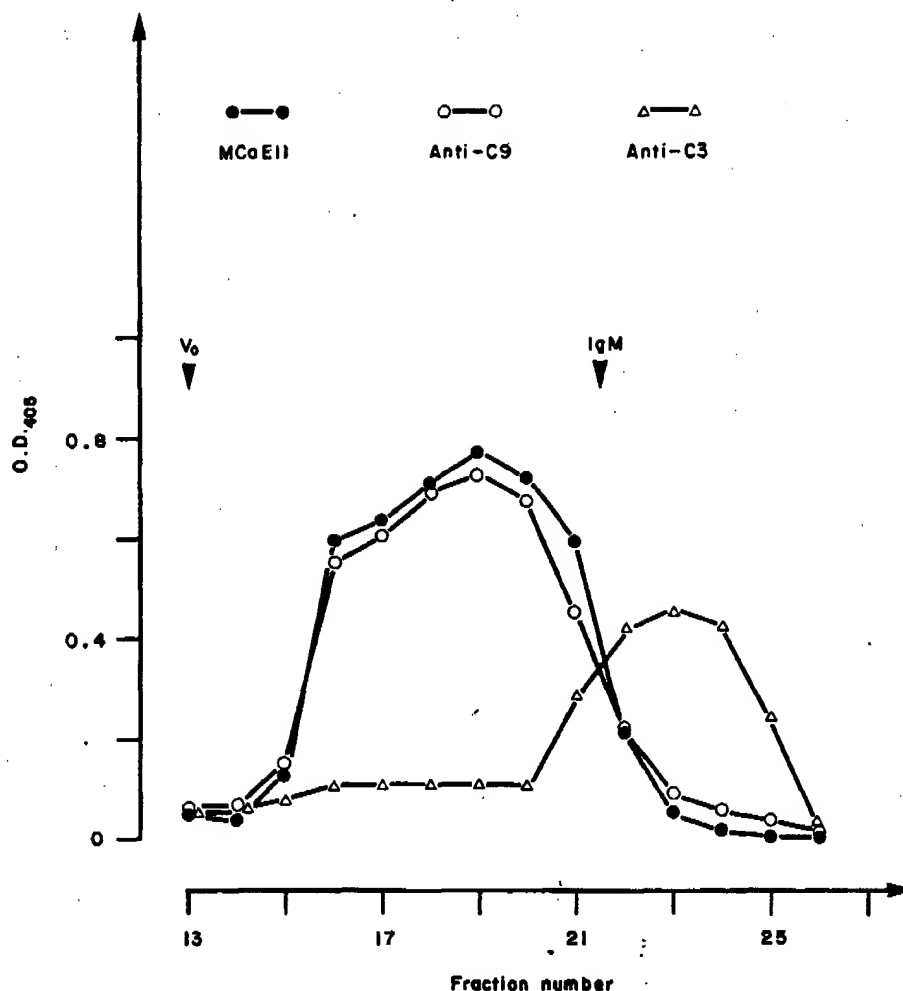


Fig. 3. Gel filtration experiments (membrane attack complex). Individual fractions obtained after passing detergent extract from complement-lysed rabbit erythrocytes through a Sephacryl S-400 column were coated onto ELISA plates and tested against MCAE11, anti-C9, and anti-C3c. The V_0 (void-volume fraction) and position of IgM are indicated with arrowheads.

the figure, which also shows that the MCAE11 peak, corresponding to the SC5b-9 complex of approximately 1000 kD in activated serum, now has changed position and appears as a more heterogeneous peak with a higher molecular weight, corresponding to the MAC.

Crossed immunoelectrophoresis. The native components C5, C6, C7, C8, and C9 and the TCC were identified in immunoelectrophoresis and CIE in the following manner. First, antibodies against each of the native components were used to identify the precipitation lines of the native components in normal plasma [18]. When we used activated serum an additional line, corresponding to the SC5b-9 complex, appeared in the alpha region [2] with each

of the five antisera used. Second, [125 I]C6 was added to a serum pool, and the mixture was activated with zymosan. Autoradiography showed radioactivity in the precipitation lines corresponding to TCC precipitated with anti-C5, anti-C6, anti-C7, anti-C8, and anti-C9. In addition, radioactivity was seen in the native C6 line. We then added [125 I]MCAE11 to antibodies against the native components, and autoradiography of CIE with activated serum showed radioactivity in the line corresponding to the TCC for all the five antibodies, whereas the native component lines were negative. [125 I]MC anti-IgM did not react with the TCC line but reacted with a line precipitated by a xenoantiserum against IgM. No line corresponding to

Fig. 4. Crossed immunoelectrophoresis (CIE) of zymosan was added

TCC was antibodies. To examine further, antibodies were mixed and tested in CIE. strong radioactivity

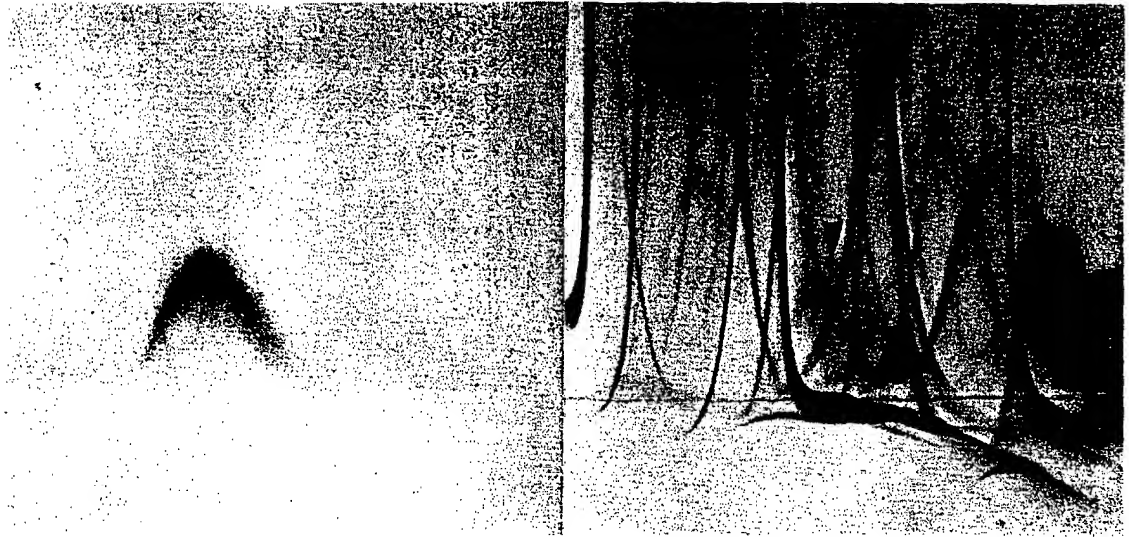


Fig. 4. Crossed immunoelectrophoresis. To the left is shown autoradiography and to the right protein staining of CIE of zymosan-activated serum precipitated by a combination of anti-whole human serum and anti-C6, to which was added [125 I]MCAE11. Arrowhead indicates the TCC line in the right part of the figure.

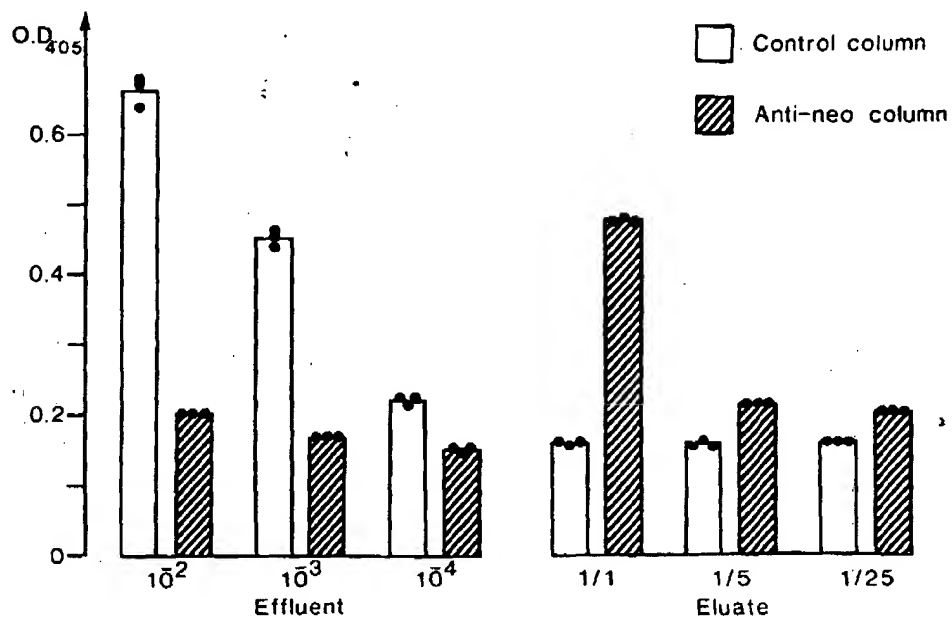


Fig. 5. MCAE11 immunosorbent. The effluents and eluates from the MCAE11 immunosorbent (anti-neo) column and the control column (uncoupled Sepharose 4B) were tested in a double-antibody ELISA with the combination of anti-C6 and anti-C5. Each column represents the mean of triplicates.

TCC was seen when we used anti-C3c antibodies.

To examine the specificity of MCAE11 further, anti-C6 and anti-whole human serum were mixed, and [125 I]MCAE11 was added and tested in CIE against activated serum. Only one strong radioactive peak was seen on the auto-

radiography (Fig. 4) corresponding to the TCC line identified by [125 I]C6, whereas this line was absent when we used normal human plasma instead of activated serum.

MCAE11 immunosorbent. Zymosan-activated serum was passed through the MCAE11 immunosorbent column and through a control

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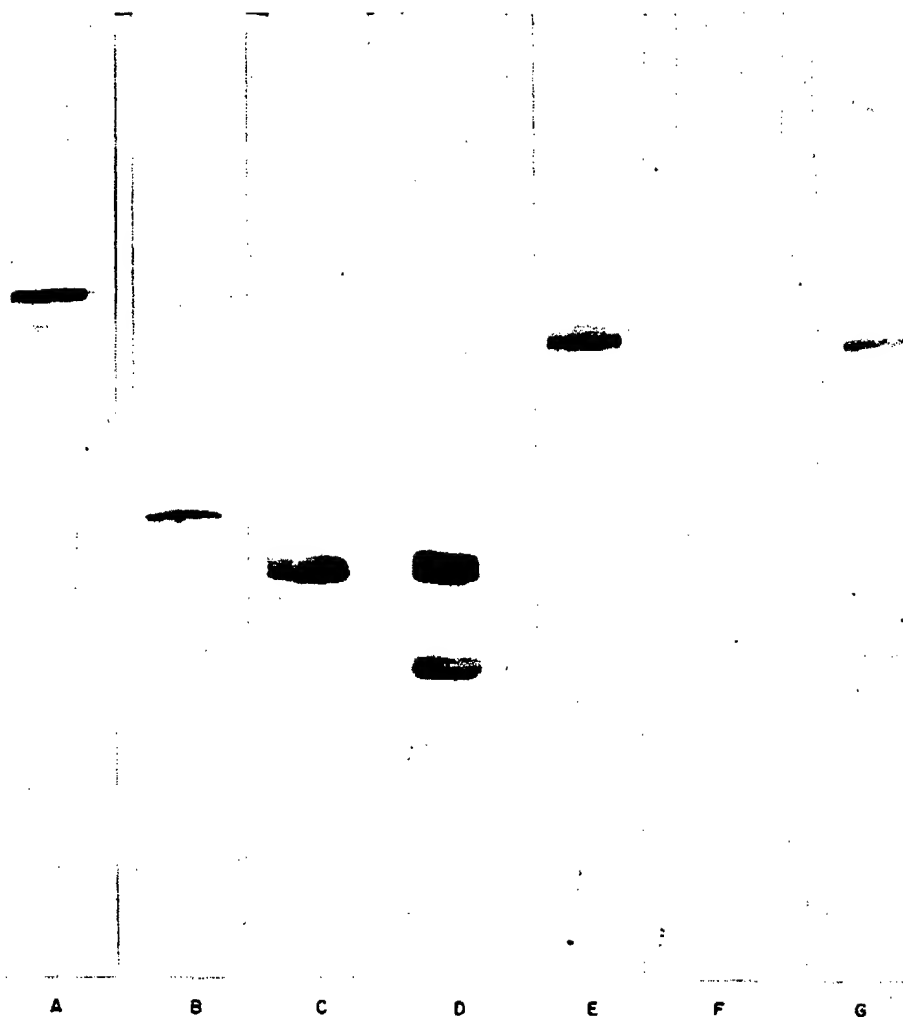


FIG. 6. MCAE11 immunosorbent. The eluate from the MCAE11 immunosorbent was examined by SDS-PAGE and subsequent immunoblotting and tested with: (A) anti-C5; (B) anti-C6; (C) anti-C7; (D) anti-C8; (E) anti-C9; (F) MCAE11; and (G) MCbC5. The line in E corresponds to dimeric C9.

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column (uncoupled Sepharose 4B). The effluents and eluates were then examined in a double-antibody ELISA with the combination of anti-C6 and anti-C5 as described earlier [17]. Fig. 5 shows that the effluent from the control column contained large amounts of TCC compared with the effluent from the MCAE11 column. In contrast, the eluate from the MCAE11 immunosorbent contained TCC, whereas the control eluate did not. The eluate from the MCAE11 column was then examined in SDS-PAGE with subsequent electroblotting onto nitrocellulose. Fig. 6 shows that it contained C5, C6, C7, C8, and dimeric C9. The bands correspond to the individual native components, as described earlier [24]. The MCbC5 showed reac-

tivity against bands corresponding to monomeric and dimeric C9, whereas the MCAE11 did not react with any of these proteins.

Subunit within the TCC binding MCAE11 and MCbC5. The binding of the two monoclonal antibodies to the purified components of the TCC was investigated. Fifty nanograms of purified C5, C6, C7, C8, C9, and poly(C9) were adsorbed onto nitrocellulose. Both antibodies, MCAE11 and MCbC5, reacted with poly(C9) and, to a much lesser extent, with C9 (Fig. 7A). None of the antibodies recognized C5, C6, C7, or C8. In a western blot analysis, in which the antigens are first denatured by SDS and then electroblotted onto nitrocellulose, only MCbC5

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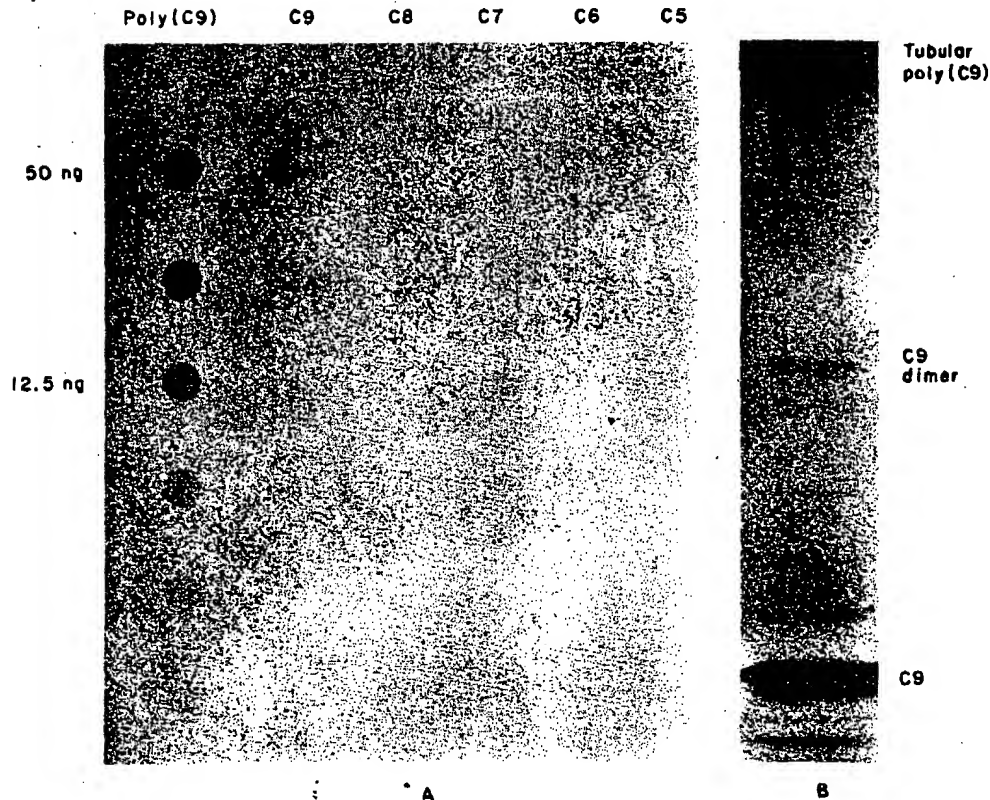


FIG. 7. Subunit of MAC interacting with the monoclonal antibodies. Purified C5, C6, C7, C8, C9, and poly(C9) (50 ng each and serial twofold dilutions thereof) were adsorbed as dots on nitrocellulose and stained, with MCAE11 (panel A) as first antibody. Similar results were obtained with MCbC5 (data not shown). Panel B shows a western blot analysis of poly(C9). Both tubular and non-tubular forms of poly(C9) are stained with MCbC5. MCAE11 did not react in a western blot analysis.

reacted with C9 and poly(C9) (Fig. 7B). In this type of analysis, poly(C9) is very inefficiently transferred to nitrocellulose owing to its high molecular weight. Staining of the blot with Ponceau S indicated that at most 5% of C9 on the blot was SDS-resistant tubular poly(C9), although initially tubular poly(C9) content amounted to 50% (data not shown). The small amount of poly(C9) was recognized by MCbC5 antibody much better than monomeric C9. Thus, immunostaining revealed a considerable fraction (30%) of C9 in its poly(C9) form.

Isotypes. The original clones MCAE11 and MCbC5 were subcloned, and ascitic fluid from the subclones were tested in agarose electrophoresis. They all showed one protein band at the same position as in the original clone. In double diffusion in gel MCAE11 was precipitated by anti-IgG2a, and MCbC5 was precipitated by anti-IgG2b.

Immunohistochemistry

A kidney section with granular deposits of IgG, IgA, IgM, and C3 was selected for examination with MCAE11. The patient was an 18-year-old woman with systemic lupus erythematosus including glomerulonephritis. A strong fluorescence was obtained with MCAE11 in the glomeruli (Fig. 8). The fluorescence was completely abolished after the ascitic fluid had been absorbed on complement-lysed rabbit erythrocytes, whereas no reduction in the fluorescence intensity was seen after absorption on mechanically lysed erythrocytes. Addition of unlabelled MCAE11 to FITC-conjugated MCAE11 showed a dose-dependent inhibition of the glomerular fluorescence. Control ascitic fluid from the cell line X63-Ag 8.653 was completely negative. Normal kidney examined after autopsy with MCAE11 showed a scattered weak



Fig. 8. MCAE11 in immunohistochemistry. Immunofluorescence obtained with MCAE11 and FITC-conjugated rabbit anti-mouse Ig in a kidney with pathologically deformed glomeruli and granular deposits of immunoglobulins and C3.

fluorescence in tubuli, but the glomeruli were negative.

ELISA assay for the TCC in plasma

All the antibodies against neoantigens of the TCC described so far detect the MAC but not its fluid-phase analogue. Recently, however, a fluid-phase TCC has been described in normal human plasma by a double-antibody ELISA based on anti-C6 and anti-C5 [17]. This assay requires fractionation of the plasma samples, to separate the TCC from the individual native components. Since this assay detects the TCC independent of the neoantigens, it was used to demonstrate the TCC in the eluate from the MCAE11 immunosorbent (see above). We now used this assay as the basis for constructing a new assay based on the MCAE11 antibody.

Anti-C6 antibodies were coated onto the plates. The Sephacryl S-300 void-volume fractions of zymosan-activated serum and normal EDTA plasma were added, and MCAE11 was used in the second antibody step. There was a strongly positive reaction with activated serum

compared with normal plasma. MC anti-IgM in the second antibody step was negative.

Alternatively, the IgG fraction of MCAE11 was used in the first step with MC anti-IgM and normal mouse IgG as control antibodies. Zymosan-activated serum and normal EDTA plasma were now tested unfractionated, and anti-C5 and anti-C9 were used in the second antibody step. MCAE11 reacted strongly with activated serum compared with normal plasma, whereas the controls were negative.

Although the reactivity against normal human plasma was low compared with activated serum, it was considerably higher than the background, and it was possible to construct and standardize an assay for quantification of the TCC in plasma, as described [16].

DISCUSSION

The main advantage of the monoclonal anti-TCC neoantigen antibodies described in this paper, compared with the ones described earlier and polyclonal xenoantisera, is that they, in

addition to detect the EDTA plasma bodies shown xenoantiserum because of the native to obtain [1, 17].

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The evaluation of bodies descriptions. (1) marked differences in zymosan-activated plasma. (2) when we compared zymosan-activated against a 1 approximate correspond was also described bodies. (4) containing lysed erythrocytes against peak was molecular weight. Thus, it is directed against the relative and in the Crossed in radiography typically. (

addition to detecting the MAC in tissue, also detect the fluid-phase TCC in normal human EDTA plasma. In general, monoclonal antibodies should be chosen rather than polyclonal xenoantisera against neoantigen of the TCC because complete absorption of activity against the native components in the complex is difficult to obtain without loss of specific activity as well [1, 17].

Several hundred clones were screened, and finally we ended up with two monoclonal antibodies, specific for a neoantigen of the poly(C9) moiety of the TCC. It must be emphasized that neoantigen is a general term, and it should not automatically be associated with the TCC. During the screening we found several antibodies against neoantigens on smaller proteins, suggested to be split products, and these were excluded after the gel filtration experiments. Thus, our screening system is a screening for antibodies against neoantigens in general, and not necessarily TCC neoantigens. However, since only a few clones were selected from the initial screening, examination of the specificity of these clones could be performed in detail.

The evaluation of the specificity of the antibodies described is based on the following observations. (1) The initial screening showed a marked difference between the activity against zymosan-activated serum and normal EDTA plasma. (2) This difference was also observed when we compared zymosan-treated serum and zymosan-treated EDTA plasma, indicating that this activity was not related to unspecified zymosan effects. (3) The activity was directed against a protein with a molecular weight of approximately one million in activated serum, corresponding to the SC5b-9 complex, which was also defined by the anti-C5 and anti-C9 antibodies. (4) Activity was observed in fractions containing MAC extracted from complemented erythrocyte membranes identified by antibodies against the native components, and this peak was heterogeneous with regard to the molecular weight as described earlier [5, 19, 26]. Thus, it is suggested that the antibodies were directed against a neoantigen expressed both in the relatively homogeneous SC5b-9 complex and in the more heterogeneous MAC. (5) Crossed immunoelectrophoresis with autoradiography expressed the specificity spectrophotically. (6) The eluate obtained from the

MCaE11 immunosorbent was positive in the anti-C6/anti-C5 double-antibody ELISA, which detects the TCC independent of anti-neoantigen antibodies. (7) That TCC had been isolated on the MCaE11 immunosorbent was suggested by the occurrence of the individual terminal components in the eluate examined by SDS-PAGE and immunoblotting. The MCaE11 did not, however, react with any of the native components.

Neoantigens were observed to appear during MAC assembly in the C5b-8 [13] and in the poly(C9) portion of the molecule [25]. Both MCaE11 and MCbC5 are binding epitopes within C9. This supports the notion that poly(C9) is part of the membrane attack complex [25]. MCaE11 was superior with regard to its ability to distinguish between monomeric and poly(C9). This antibody most likely reacts with a domain within poly(C9) which is destroyed during SDS solubilization. In contrast, MCbC5 recognizes a segment of amino acids which is also exposed in denatured monomeric C9. Since MCbC5 inhibits binding of MCaE11 to poly(C9) (data not shown), their epitopes must be close. MCbC5 binds to amino acids located in the middle of C9's polypeptide chains (K. Stanley *et al.*, *EMBO Journal*, 4, 375, 1985).

It seems apparent that most monoclonal anti-TCC neoantigen antibodies, including the one described by Falk *et al.* [7], are directed towards C9. This shows that major conformational changes occur within this molecule during MAC assembly. In fact, electron microscopic examination of monomeric and poly(C9) showed major differences, including an increase in length of C9 from 8 to 16 nm [28]. Similar new antigenic sites have to be exposed within C9 on the formation of fluid-phase TCC, since the monoclonal antibodies detect an antigenic determinant of the MAC and of the SC5b-9 complex. C9 polymerization has been reported to be inhibited in SC5b-9 [23]. Our results, however, suggest that C9 polymerization and its concomitant rearrangement occurs in this complex. This is corroborated by the presence of disulphide-linked C9 dimers [31] within the SC5b-9 (see Fig. 6).

It is now generally accepted that demonstration of MAC in tissue indicates local complement activation [3]. Demonstration of a neoantigen of poly(C9) also indicates that the whole terminal pathway has been activated.

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Several patients with evidence of complement activation have been found to have increased amounts of TCC in plasma. Thus, since MCAE11 can be used for evaluation of terminal pathway activation both in tissue and in plasma, it may be one of the most important tools with which to examine further the role of terminal pathway activation and membrane attack mechanism in the physiology and the pathophysiology of complement. The only monoclonal antibody against neoantigen of poly(C9) described earlier [7] could be used for detection of MAC in tissue, whereas the fluid-phase TCC could not be detected. This is an important additional application for MCAE11. We suggest that our procedures for immunization, screening, and detection of the monoclonal antibodies favour selection of a population of antibodies against neoantigens ranging from highly specific to less specific. This is in contrast to what would be expected from the procedure described by Falk et al. [7]. They immunized with a heterogeneous extract from pathological kidneys, and their screening system was not based on selection of antibodies against neoantigens.

It still remains to be shown to what extent the circulating TCC in human plasma may reflect the lytic activity of complement in tissue and, more generally, how disease activity is related to activation of the terminal pathway in vivo. Assays for the TCC, both in tissue and in body fluids, are suggested to be an important supplement to already established methods for evaluation of complement activation, which mostly detect activation of early components. Demonstration of the TCC, however, specifically indicates activation of the terminal pathway. Thus, by quantification of, for example, C3 split products and the TCC, it may be possible both to compare and to distinguish between activation of the early and the late phase of the complement cascade in vivo. Monoclonal antibodies against neoantigens of the TCC are of obvious importance in work with these problems and for further characterization of the MAC assembly and the membrane damage mechanisms.

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Monoclonal antibodies against neoantigens of the terminal C5b-9 complex of human complement

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Assembly of the terminal C5b-C9 complement components into the cytolytic C5b-9 complex is accompanied by exposure of characteristic neoantigens on the macromolecule. We report the production and characterization of mouse monoclonal antibodies to C9-dependent neoantigens of human C5b-9. Binding-inhibition assays with EDTA-human plasma and micro-ELISA assays with purified C9 showed that the antibodies did not react with native complement components and thus confirmed the specificity of the antibodies for the neoantigens. The monoclonal antibodies did, however, cross-react with cytolytically inactive, fluid-phase C5b-9 complexes. Thus, expression of the neoantigenic determinants was not dependent on the formation of high molecular weight C9 polymers with the complex, since these are absent in fluid-phase C5b-9. Radioiodinated antibodies could be utilized in immunoradiometric assays for the detection and quantitation of C5b-9 on cell membranes. Cross-reactivities of the antibodies with C9-dependent neoantigens of several other animal species were examined and antibody clones cross-reacting with rabbit (clones 3B1, 3D8, and 2F3), sheep (clones 3D8 and 2F3) and guinea-pig (clone 3D8) neoantigens were identified. Three of four tested clones (3D8, 2F3, 1A12) precipitated C5b-9 complexes in double-diffusion assays, probably due to their interaction with multiple and repeating C9-epitopes on the terminal complexes. The monoclonal antibodies will be of value for definitive identification and quantitation of C5b-9 on cell membranes and in tissues, and for establishing immunoassays for detection and quantitation of terminal fluid-phase C5b-9 complexes in plasma.

Assembly of plasma complement components C5-C9 into macromolecular C5b-9 complexes constitutes the terminal reaction of the complement cascade (Bhakdi & Trantum-Jensen, 1983). When occurring

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on a target lipid bilayer, this reaction results in the formation of heterogeneous C5b-9 complexes composed mainly of C5b-8 monomers to which varying numbers (2-16?) of C9 molecules are bound (Bhakdi & Trandum-Jensen, 1981; Tschopp et al., 1983; Bhakdi & Trandum-Jensen, 1984). Complexes harbouring high numbers of C9 (8-16?) exhibit the structure of hollow protein cylinders that lie partially embedded within (Bhakdi, 1983). The major (Podack et al., 1983) albeit not sole (Bhakdi et al., 1980) constituent of the intramembranous channel is C9, which appears to span the entire bilayer (Morgan et al., 1984). Complexes carrying lower numbers of C9 also produce smaller hydrophilic transmembrane channels, the structures of which have not been described. Channel formation by C5b-9 is generally regarded as the primary mode through which complement exerts its membrane-damaging effects (Bhakdi & Trandum-Jensen, 1983; Mayer et al., 1981).

Complement can also be activated in the fluid-phase, e.g. by the presence of immune complexes or activators of the alternative complement pathway in serum (Kolb & Müller-Eberhard, 1975). This can lead to the generation of a cytolytically inactive, water-soluble C5b-9 complex, termed SC5b-9 (Kolb & Müller-Eberhard, 1975a; Bhakdi & Roth, 1981) or, as recently proposed, XC5b-9 (Jenne et al., 1985). Both the fluid-phase and the membrane C5b-9 complexes carry characteristic neoantigens that are not expressed on the native C5-C9 proteins (Kolb & Müller-Eberhard, 1975b; Bhakdi et al., 1978; Bhakdi & Muhly, 1983; Bhakdi et al., 1983). The bulk of neoantigens derives from C9 (Podack et al., 1982). The neoantigens are of practical importance since they represent very reliable and stable immunological markers for the terminal complex (Bhakdi & Trandum-Jensen, 1983; Biesecker, 1983). In this communication, we report the production and characterization of mouse monoclonal antibodies to C9-dependent neoantigens of human C5b-9. The antibodies can be utilized in all standard immunoassays. They can be used to identify C5b-9 in tissues, to quantify C5b-9 on cell membranes, and will also permit development of simple immunoassays for fluid-phase C5b-9 complexes in plasma.

Materials and Methods

Complement-lysed rabbit erythrocyte membranes were prepared as described elsewhere (Bhakdi et al., 1983) and trypsinized (50 µg/ml final enzyme concentration; approx. 10 mg/ml membrane protein in the washed membrane pellets) for 30 min, 37°C. The membranes were then solubilized in 250 mM deoxycholate, and the protease-resistant C5b-9 complex was isolated by a single ultracentrifugation step through a linear sucrose density gradient containing 6.25 mM deoxycholate (Bhakdi et al., 1983). Fractions containing the complex were pooled and used as antigen for immunization and for micro-ELISA assays.

Monoclonal antibodies

Female C 57 Bl/6J mice (Zentralinstitut für Versuchstiere, Hannover, GFR) were injected with 100 µg of protein per immunization. For primary immunization, antigen solutions were admixed with 1 vol. of Freund's complete adjuvant and injected in both hind

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footpads. Booster immunization was performed 4 weeks later by subcutaneous injections of the antigen in incomplete adjuvant. Final immunizations were performed 3-5 weeks thereafter following a published protocol (Stähli et al., 1980) with the antigen applied without adjuvant i.p. and i.v. 4 d prior to fusion of the cells.

Cells of the myeloma line X 63-Ag 8.6.5.3. cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml) were fused with the spleen cells derived from immunized mice in the conventional manner (Köhler & Milstein, 1975; Galfré & Milstein, 1981) using 50% PEG as the fusing agent. Fused cells were diluted in HAT-containing RPMI 1640 (0.1×10^{-2} mM hypoxanthine, 0.04×10^{-2} mM aminopterin, 1.6×10^{-2} mM thymidine) and distributed into 4 x 96 Nunc tray wells. Hybrid cultures producing specific antibodies were cloned thrice by limiting dilution (Levkovits & Waldmann, 1979; de Blas et al., 1983). Large scale production of monoclonal antibodies was carried out in culture plates of 14 cm diameter. Approx. 1000 ml of culture supernatants were concentrated tenfold (Amicon concentration chamber) and the immunoglobulins precipitated by addition of sodium sulfate (18% w/v final concentration). Final purification of the antibodies was achieved with the use of Affi-Gel Protein A (MAPS, Bio-Rad Lab., Munich, GFR). The purity of the immunoglobulin preparations was checked by SDS/PAGE. The purified immunoglobulin preparations (0.5-0.8 mg/ml protein) were utilized in various immunoassays and radioiodinated for use in immunoradiometric assay (IRMA) as described (Bhakdi & Kayser, 1981).

Routine screening assays for antibody production by the hybridoma cells were performed using purified C9 (isolated from human serum according to Biesecker and Müller-Eberhard, 1980), C5b-9, and 'poly-C9' (isolated according to Tschopp, 1984) as antigens in micro-ELISA-assays. Antibodies specific for the C9-dependent neoantigens characteristically reacted strongly with C5b-9 and 'poly-C9', but at most only very weakly with native C9 in these tests. Clones which were positive with C5b-9 but negative with 'poly-C9' were tested by line immunoelectrophoresis combined with immunoblotting using a recently described technique (Bhakdi et al., 1985) which permitted immediate identification of C5-C9 specificities.

For the analysis of antigenic cross-reactivities amongst C9-dependent neoantigens from different animal species, rabbit erythrocytes were first treated with C8-depleted human serum to generate C5b-7 cells as described elsewhere (Bhakdi & Muhly, 1985). The washed cells were resuspended in saline / 5 mM EDTA, pH 7.4 (10^9 cells/ml) and post-treated with 1 vol. of EDTA-plasma from rabbit, mouse, rat, sheep, guinea-pig or chicken. Cell lysis ensued in all cases, indicating binding of C8 and C9 from the heterologous animal plasma to human C5b-7. As controls, C5b-7 cells were given purified human C8 with or without C9 (10^6 molecules/cell), or with 1 vol. of EDTA human serum. The membranes were subsequently washed thrice in saline and utilized in radioassays.

Results and Discussion

Individual antibody clones specific for C5b-9 neoantigens were selected during screening assays on the basis of their positive reactions

in micro-ELISA assays with purified C5b-9 and poly-C9, and their negative reactions with native C9. A monoclonal antibody specific for human C5, designated 1E8, was identified by crossed-immunoelectrophoresis-immunoblotting (Bhakdi et al., 1985). Four of eleven presently available anti-neoantigen clones, designated 3B1, 3D8, 2F3, and 1A12, were randomly selected for the present studies. The antibodies were purified from cell culture supernatants with overall yields of 60-70%. Each purified protein preparation yielded a single band of approx. M_r 160 000 in SDS/PAGE (Fig. 1), which was cleaved upon reduction into the heavy and light chains. After radioiodination, over 95% of radioactivity was recovered in these bands (Fig. 1). All four antibody clones described in this paper were of subclass IgG 2b as determined by ELISA using peroxidase conjugated, affinity purified rabbit anti-mouse subclass specific antibodies (Miles Scientific, Naperville, U.S.A.).

Fig. 2 depicts the results of direct immunoradiometric assays that were representative of all four clones. Complement-lysed sheep erythrocyte membranes (2×10^9 cells/ml) in saline / 1% BSA were doubly-diluted (100 μ l aliquots) and incubated with radiolabelled antibody (100 μ l, approx. 20 000 c.p.m./vial), for 60 min, 22°C. The cells were washed thrice and bound radioactivity was measured and expressed as percent of total original radioactivity in the vial (Bhakdi & Kayser, 1981). Maximal binding values of 60-75% were registered under these conditions (Fig. 2). The detection limit for C5b-9 in

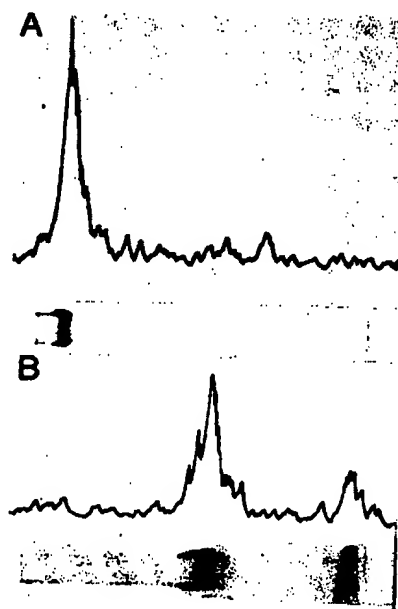


Fig. 1. SDS/PAGE of radiiodinated, purified monoclonal antibody (clone 3B1) to C5b-9 neoantigen. The Coomassie-stained gels and the corresponding radioactivity scans are shown. A, unreduced; B, reduced sample showing cleavage of the IgG into heavy and light chains.

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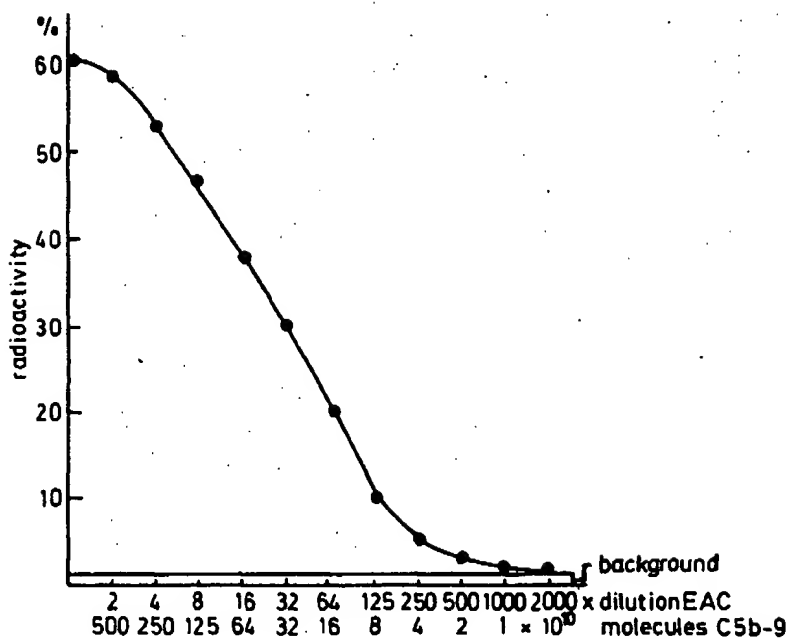


Fig. 2. Binding of radioiodinated monoclonal anti-C5b-9 antibodies (clone 3B1) to complement-lysed erythrocyte membranes. A suspension of membranes was doubly diluted and immunoradiometric assays performed as described in Materials and Methods. These assays permitted detection of approx. 10^{10} molecules of C5b-9 in a sample.

these assays was approximated as follows. The concentration of C5b-9 in the starting membrane suspensions was determined to be 0.2 ± 0.05 mg/ml by rocket immunoelectrophoresis of solubilized membranes and using a purified C5b-9 solution as standard (1 mg/ml, determined by amino acid analysis). Taking a mean molecular weight for C5b-9 of 1.12×10^6 (Bhakdi & Tranum-Jensen, 1984), this corresponded to approx. 10^{14} molecules C5b-9/ml membrane suspension, or 10^{13} molecules in a 100 μ l aliquot. The detection limit of the radioassay was thus of the order of 10^{10} molecules C5b-9 per probe. Since probe volumes could be increased to 1 ml (2×10^9 erythrocytes), the assay should permit detection of an average of 5-20 C5b-9 molecules per cell.

Binding inhibition assays confirmed the specificity of the antibodies for C5b-9 neoantigens. In these experiments, radioiodinated antibodies were pre-incubated with 10 μ l of 100 μ l of EDTA-plasma for 60 min, 22°C. Thereafter, the complement-treated membranes were added, incubations were continued for another 60 min, and bound radioactivity was subsequently measured. Characteristically, binding-inhibition of less than 12% occurred in the presence of 100 μ l undiluted EDTA-plasma. In contrast, parallel analyses performed with monoclonal anti-C5 antibodies (clone 1E8) showed marked reduction in binding of the radiolabelled antibodies (over 50% binding inhibition in the presence of 100 μ l of EDTA-plasma; Fig. 3). The binding

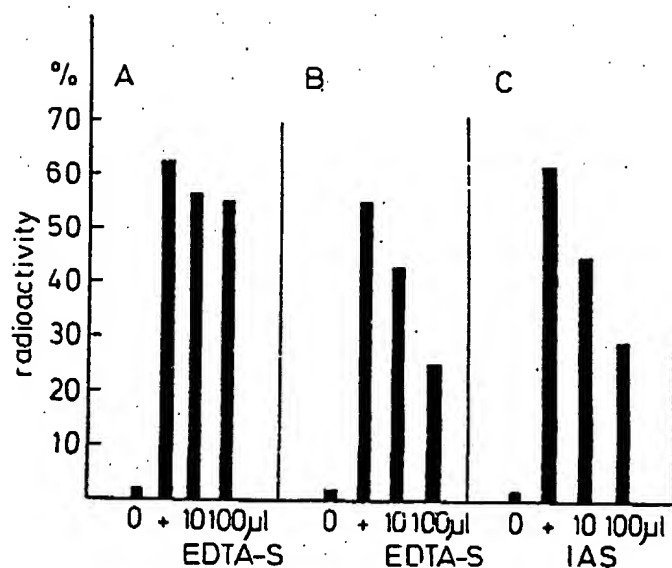


Fig. 3. Immunoradiometric assays with a radio-iodinated, monoclonal anti-neoantigen antibody (A and C), and with a monoclonal antibody to C5 (B). Complement-lysed erythrocyte membranes were offered as binding substrate to aliquots of the antibodies and binding was expressed as % of total radioactivity as described in Materials and Methods. (A) Inhibition of binding elicited through pre-incubation of anti-neoantigen antibodies (clone 3B1) with EDTA-serum: 0 = negative control membranes; + = positive control membranes carrying C5b-9; 10 and 100 µl columns depict the binding values obtained upon pre-incubation of the antibodies with the given volumes of EDTA-serum. Only very weak binding-inhibition was observed, indicating non-cross-reactivity of the antibodies with native complement components. (C) The same experiment, conducted with inulin-activated serum (IAS) as a source for XC5b-9. Marked binding-inhibition occurred, demonstrating the reaction of the antibodies (clone 3B1) with XC5b-9. (B) Same experiments as in A, conducted with monoclonal anti-C5 (clone 1E8) to demonstrate the binding-inhibition obtained with EDTA-serum when the antibody was directed against a native C5-C9 antigenic determinant.

behaviour of monoclonal anti-neoantigen antibodies was virtually identical to that previously observed with radiolabelled, polyclonal rabbit antibodies (Bhakdi & Muhly, 1983; Bhakdi et al., 1983). The slight binding inhibition elicited at very high concentrations of EDTA-plasma may be due to very small amounts of fluid-phase C5b-9 complexes present in normal plasma (Mollnes et al., 1984; Falk et al., 1985; Hugo & Bhakdi, unpublished data).

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In contrast to the observations made with EDTA-plasma, pre-incubation of anti-neoantigen antibodies with inulin-activated serum containing fluid-phase XC5b-9 caused marked reduction in antibody-binding to the membranes (Fig. 3). This again conformed to previous data obtained with polyclonal rabbit antibodies (Bhakdi & Muhly, 1983) and showed that the monoclonal antibodies also reacted with fluid-phase complexes. Positive results were also obtained in micro-ELISA assays using XC5b-9 as antigen (not shown). The finding that monoclonal anti-neoantigen antibodies reacted with XC5b-9 was of interest since these complexes do not contain high molecular weight 'poly-C9' (Bhakdi & Trandum-Jensen, 1983); thus, expression of neoantigenic activity is obviously not dependent on the formation of these C9-polymers, and is elicited already through the binding of low numbers of C9 to C5b-8. It is known that the cytolytically inactive, fluid-phase XC5b-9 complexes harbour only 2-3 molecules C9 per complex (Bhakdi & Trandum-Jensen, 1983).

The next experiments were designed to test the stage in C5b-9 assembly at which neoantigens detectable with the present antibodies appeared, and to determine possible cross-reactions amongst C5b-9 neoantigens of heterologous species. Rabbit erythrocytes carrying human C5b-7 were post-treated with an excess of C8 or C8 + C9. Radioassays performed with three monoclonal anti-neoantigen antibodies confirmed that the neoantigens appeared only after binding of C9 (Fig. 4A). The antibodies selectively cross-reacted with neoantigens generated by binding of heterologous C8/C9 to human C5b-7 (Fig. 4B-4D). One antibody clone (clone 3B1, Fig. 4B) strongly cross-reacted only with rabbit C9-neoantigen. The second clone (3D8, Fig. 4C) bound avidly to complexes carrying rabbit C9-neoantigens. Intermediate binding to sheep and weak binding to guinea-pig neoantigens was observed. The third clone (2F3, Fig. 4D) reacted strongly with rabbit, and weakly with sheep neoantigens. None of the tested clones reacted with neoantigens of rat, mouse or chicken C9. The weak binding observed in several cases probably derived from low-affinity interaction of these antibodies with the heterologous antigens. The described simple procedure permits rapid identification of species cross-reactivities of the monoclonal antibodies, and appropriate clones may thus be selected for use in animal model studies. The analyses also indicated that the three tested clones were each directed against a distinct neoantigenic determinant.

A protein antigen carrying multiple, repeating epitopes may be expected to be precipitable by monoclonal antibodies. This was indeed found with three of the four tested anti-neoantigen clones, namely 3D8, 2F3, and 1A12, whereas precipitation of human C5b-9 was not observed with clone 3B1 in double-diffusion analyses. No immunoprecipitates were formed when EDTA-plasma or inulin-activated plasma was applied as antigen (results not shown).

No reaction of any neoantigen specific monoclonal antibody was discerned in SDS/PAGE immunoblotting of C9 applied either as native purified antigen or derived from whole human plasma. Thus, SDS-denaturation of C9 does not lead to 'exposure' of neoantigenic determinants in the molecule that react with the present monoclonal antibodies. However, very weak positivities were observed with undenatured C9 in micro-ELISA assays with some antibodies, and these

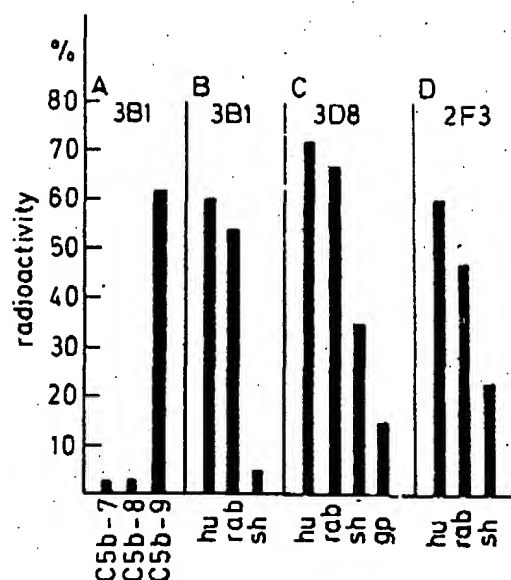


Fig. 4. Specificity and species cross-reactivities of monoclonal anti-neoantigen antibodies. (A) Binding of radiolodinated antibodies (clone 3B1) to cells carrying C5b-7, C5b-8 and C5b-9: note the binding only to the latter cells. (B-D) Binding of monoclonal antibodies (clones 3B1, 3D8 and 2F3) to cells carrying human, rabbit, sheep or guinea-pig C9-neoantigens. Note the different patterns of cross-reactivities observed with the different clones.

results were similar to those obtained by Mollnes et al. (1985) for the two monoclonal anti-neoantigen antibodies obtained in their laboratory.

In summary, the presently available monoclonal antibodies against C9-dependent neoantigens of the terminal complement complex may be used in all conventional immunoassays. They permit definitive identification and localization of C5b-9 in normal and diseased tissues (unpublished data). Due to the excellent cross-reactivities of some clones with heterologous C5b-9 neoantigens, appropriate antibody clones may be selected for use in animal model studies. Immunoradiometric assays permit quantitation of C5b-9 on target cells and cell membranes. Simple micro-ELISA tests as well as radioimmunoassays have been constructed for assaying the fluid-phase terminal complex in human plasma (Mollnes et al., 1985; Falk et al., 1985; Hugo & Bhakdi, unpublished data). Identification of the C9 sequences that encode the monoclonal antibody epitopes should also be feasible. One neoantigenic epitope has recently been localized to a buried region approximately in the middle of the C9 polypeptide chain (Stanley et al., 1985).

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Neoantigen of the Polymerized Ninth Component of Complement

CHARACTERIZATION OF A MONOCLONAL ANTIBODY AND IMMUNOHISTOCHEMICAL LOCALIZATION IN RENAL DISEASE

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ABSTRACT A monoclonal antibody to a neoantigen of the C9 portion of the membrane attack complex (MAC) of human complement has been developed and characterized. The distribution of this neoantigen was assessed by indirect immunofluorescence microscopy in nephritic and nonnephritic renal diseases. The antibody (Poly C9-MA) reacted on enzyme-linked immunosorbent assay (ELISA) with a determinant in complement-activated serum that was undetectable in normal human serum (NHS). Zymosan particles incubated in NHS had positive immunofluorescent staining with Poly C9-MA; however, binding of Poly C9-MA was not observed with zymosan particles incubated in sera deficient in individual complement components C3, C5, C6, C7, C8, or C9. Reconstitution of C9-deficient sera with purified C9 restored the fluorescence with Poly C9-MA. Poly C9-MA reacted positively by ELISA in a dose-dependent manner with purified MC5b-9 solubilized from membranes of antibody-coated sheep erythrocytes treated with NHS but not with intermediate complement complexes. Poly C9-MA also reacted in a dose-dependent manner on ELISA and in a radioimmunoassay with polymerized C9 (37°C, 64 h) (poly C9) but not with monomeric C9. Increasing amounts of either unlabeled poly C9 or purified MC5b-9 inhibited the ¹²⁵I-poly C9 RIA in an identical manner. These studies demonstrate that Poly C9-MA recognizes a neoantigen of C9 common to both the MAC and to poly C9. By immunofluorescence, Poly C9-MA reacted minimally with normal

kidney tissue in juxtaglomerular loci, the mesangial stalk, and vessel walls. Poly C9-MA stained kidney tissue from patients with glomerulonephritis in a pattern similar to that seen with polyclonal anti-human C3. In tissue from patients with nonnephritic renal disease—diabetes, hypertension, and obstructive uropathy—Poly C9-MA was strongly reactive in the mesangial stalk and juxtaglomerular regions, tubular basement membranes, and vascular walls. Poly C9-MA binding was especially prominent in areas of advanced tissue injury. Poly C9-MA frequently stained loci where C3 was either minimally present or absent. These studies provide strong evidence for complement activation not only in nephritic but also in nonnephritic renal diseases.

INTRODUCTION

The complement system has been implicated in the pathogenesis of experimental and human renal disease by virtue of changes in serum levels and by the demonstration of complement components in diseased tissue (1). One of the mechanisms of complement-induced membrane damage is mediated through the assembly of terminal complement components into the membrane attack complex (MAC)¹ MC5b-9 (2). The

¹ *Abbreviations used in this paper:* DOC, sodium-desoxycholate; EA, antibody-coated sheep erythrocytes; EAC, complement-coated EA; ELISA, enzyme-linked immunosorbent assay; GBM, glomerular basement membrane; LPS, endotoxin lipopolysaccharide; MAC, membrane attack complex; MPGN, membranoproliferative glomerulonephritis; NHS, normal human serum; poly C9, polymerized C9; Poly C9-MA, monoclonal antibody to a neoantigen of C9; SLE, systemic lupus erythematosus; VBS, veronal-buffered saline.

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five MAC precursor proteins assemble after cleavage of complement component C5 by classic or alternative complement pathway activation (3). Once formed, the MAC binds with high affinity to phospholipid binding sites on cell membranes (4). Presumably, the MAC is capable of physically inserting itself into and then reorganizing membrane lipid bilayers (5, 6). In the process of assembly of the MAC, the terminal complement components undergo conformational changes exposing neoantigenic structures in C5b6 and in C5b67, recognized by previously described polyclonal antibodies (7). The C5b67 complex is membrane bound, allowing for subsequent C8 and C9 attachment (6). Recently, Podack et al. (8) have demonstrated that polymerization of C9 induced by cell-bound C5b-8 or by prolonged incubation of monomeric C9 at 37°C forms tubular structures with an ultrastructural appearance that resembles that of the isolated MAC. Additionally they have produced a polyclonal antiserum to a neoantigen(s) on polymerized C9 (poly C9) that is similar to the antigen(s) present on isolated MAC (9).

Although the MAC has been recognized for several years on lymphocytes (10), polymorphonuclear leukocytes (11), and platelets (12), its presence in diseased tissues has been appreciated only recently. Biesecker et al. (13, 14) demonstrated that a polyclonal antibody to MAC fixes to immune deposits in the kidney and skin of patients with systemic lupus erythematosus (SLE).

In the course of studies of monoclonal antibodies to human renal basement membranes, we found and characterized a monoclonal antibody (Poly C9-MA) to a neoantigen present in the C9 portion of the MAC. In normal kidney tissue, this antigen is present in small vessels and minimally within the glomerular stalk, whereas in kidney tissue from a variety of diseases there is extensive deposition of this neoantigen of the MAC.

METHODS

Buffers. The following buffers were used: Veronal-buffered saline (VBS); VBS containing 0.15 mM CaCl_2 and 0.5 mM MgCl_2 (VBS⁺⁺); phosphate-buffered saline (PBS) (0.15 M NaCl, 0.01 M NaH_2PO_4 , pH 7.4); 0.5 PBS (0.5 M NaCl, 0.01 M NaH_2PO_4 , pH 7.4); PBS-EDTA (PBS with 10 mM EDTA); BEP buffer (5 mM Na borate with 10 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, pH 8.8) (15); enzyme-linked immunosorbent assay (ELISA) coating buffer (0.1 M Na_2CO_3 , pH 9.6); ELISA wash buffer (0.5 PBS with 0.05% Tween 20, Sigma Chemical Co., St. Louis, MO); citrate phosphate buffer (0.5 M citric acid and 0.1 M NaH_2PO_4 , pH 5.0); and Tris-hydroxymethyl aminomethane-buffered saline (Tris-buffered saline; 0.10 M Tris, 0.15 M NaCl, pH 7.4).

Complement components and reagents. The following human complement proteins were purified as described previously: C5 (16), C6 (17), C5b6 (18), C7 (17), C8 (19), and C9 (20). C5b67 complexes were formed by reacting 100 μg of C5b6 with 50 μg of C7 for 30 min at 37°C. Poly C9 was

prepared by incubating purified C9 at 1 mg/ml for 64 h at 37°C in Tris-buffered saline containing 25 μg soybean trypsin inhibitor (8). Electron microscopic analysis of the poly C9 using a negative staining technique showed tubular structures identical to those previously reported (8). Analysis of the purified poly C9 by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was conducted on a 2.5–10% polyacrylamide gradient as previously described (9). The purity of the preparation is demonstrated in Fig. 1. By densitometric scanning in different preparations, 22–50% of the material was present in the poly C9 form. Some experiments were performed with functionally pure C8 and C9 (Cordis Laboratories, Inc., Miami, FL).

Zymosan (Becton-Dickinson and Co., Orangeburg, NY), cobra venom factor (Naja Naja) (Cordis Laboratories), and endotoxin lipopolysaccharide (LPS) from *Escherichia coli* 026:B6 (lot 613126, Difco Laboratories, Detroit, MI) were used as complement activators. Human sera deficient in specific complement components, C1r, C2, C3, C5, C6, C7, C8 (α, γ chain), C8 (β chain), and C9 were characterized by component analysis as previously described (21). An additional C9 deficient serum was a kind gift from Dr. Shinya Inai, Osaka, Japan. Single donor normal human cryoprecipitate (22) was obtained from the University of Minnesota Blood Bank.

Collagen types I, III, IV, and V were isolated from human placenta (23), but without reduction, carboxymethylation, or repeated pepsin digestion. Laminin was a gift of Dr. Hynda Kleinman (National Institute of Dental Research), and fibronectin was a gift of Dr. Leo Furcht (University of Minnesota).

Development of the monoclonal antibody Poly C9-MA. Collagenase-digested normal human glomerular basement membrane (GBM) was prepared from kidney tissue that was obtained at the time of autopsy after accidental death (24, 25). Balb/c female mice were immunized by an intraperitoneal injection of 100 μg of GBM homogenized in complete Freund's adjuvant (Gibco Laboratories, Grand Island, NY), followed by two immunizations in PBS at intervals of 14 d.

Hybridization and cloning. 4 d after the final immunization, spleen cells in Dulbecco's modified Eagle medium

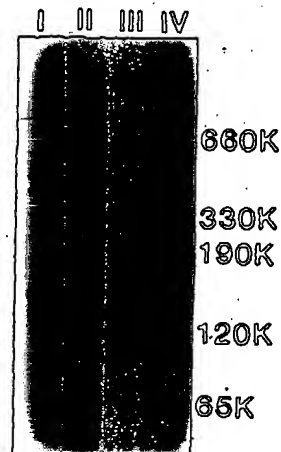


FIGURE 1 SDS-polyacrylamide gel electrophoresis (2.5–10% gradient) of purified C9 (lane I); poly C9 (lane II), purified C5 (lane III), and molecular markers (lane IV). By densitometric scanning of lane II, ~25% of purified C9 polymerized into poly C9.

(Microbiological Associates, Walkersville, MD) were fused with the Balb/c myeloma cell line P3-Ns1-Ag4-1 (obtained from Dr. Tucker LeBien, University of Minnesota) in polyethylene glycol (PEG 1000, J. T. Baker Chemical Co., Phillipsburg, NJ) (26). The cells were grown in 96-well microtiter plates (Costar, Cambridge, MA) in HAT medium containing Littlefield's concentrations of hypoxanthine, aminopterin, and thymidine (27). Supernatants from visible hybrid colonies were screened for antibody by indirect immunofluorescence on normal human kidney. Colonies of interest were subcloned by limiting dilution, then grown to culture volume, and injected into pristane-primed Balb/c female mice. Ascites fluid was harvested 7 to 14 d after injection (28). The supernatant of cell cultures was stored at -70°C .

Immunofluorescent studies. Tissue was snap frozen in isopentane precooled in liquid nitrogen and sectioned at $4\ \mu\text{M}$ in a Lipshaw cryostat (Lipshaw Manufacturing, Co., Detroit, MI). After air-drying, sections were fixed in acetone for 10 min, washed three times with PBS, overlaid with $\sim 20\ \mu\text{l}$ of an appropriate dilution of monoclonal ascites fluid, or goat antibody to human C3, C5, C6, C7, C8, or C9 (Miles Laboratories, Elkhart, IN). Each goat antiserum was absorbed with its respective complement-deficient serum before use. Monoclonal antibodies were reacted with human plasma-absorbed fluorescein-isothiocyanate-conjugated goat anti-mouse Ig (γ , μ , α -chain specific; Cappel Laboratories Inc., Cochranville, PA) and the complement component antibodies were reacted with human plasma-absorbed fluorescein-isothiocyanate-conjugated rabbit anti-goat Ig (γ , μ , α -chain specific, Cappel Laboratories) (29). The distribution and intensity of immunofluorescence was scored on a semiquantitative scale of trace, moderate, and strong fluorescence. Class-specific reagents to mouse immunoglobulin heavy and light chains (Bionetics Laboratory Products, Kensington, MD) were used after the application of the monoclonal antibody to determine Ig class specificity. Immunofluorescent studies with Poly C9-MA were performed on lymphocytes, monocytes, and polymorphonuclear leukocytes, which were obtained by Ficoll-Hypaque gradient centrifugation as previously reported (30).

Zymosan immunofluorescence assay. Zymosan was added to VBS at 1 mg/ml and boiled at 100°C for 30 min. The sample was centrifuged at 4°C and at 300 g for 5 min, resuspended and washed three times with cold VBS⁺⁺ (31). The zymosan particle suspension ($100\ \mu\text{g}$ in $100\ \mu\text{l}$) was incubated at 37°C in $100\ \mu\text{l}$ normal human serum (NHS), heat-inactivated NHS (prepared by preincubation at 56°C for 30 min), NHS containing 10 mM EDTA, or a specific complement component deficient serum, C1r, C2, C3, C5, C6, C7, C8 (α - γ chain), C8 (β chain), or C9. The zymosan particles were washed three times with cold VBS as above, air dried on glass slides, and stained by indirect immunofluorescence with goat antisera to human C3, C5, C6, C7, C8, or C9 (each absorbed with boiled zymosan particles) and Poly C9-MA. In some studies, a monoclonal antibody that reacts with human basement membrane (MBM10) of the same subclass as Poly C9-MA [IgG₁, κ] was also used. C8- or C9-deficient human serum ($100\ \mu\text{l}$) was reconstituted with the respective functional component, C8 or C9 (400 hemolytic units), and incubated with zymosan particles for 30 min at 37°C , washed, and prepared for immunofluorescence microscopy.

ELISA studies (32, 33). Antigens were incubated in 96-well microtiter plates (Costar) with ELISA coating buffer or with PBS in a moist chamber at room temperature for 3 h, then overnight at 4°C . The plates were washed three times with ELISA wash buffer and incubated at room temperature for 1 h with $100\ \mu\text{l}$ of monoclonal antibody or goat antisera

specific for human complement components (C5, C6, C7, C8, or C9) diluted in PBS. After three washes, the plates were incubated for 30 min at room temperature with human plasma-absorbed peroxidase-labeled goat anti-mouse Ig (γ , μ chain specific) (Tago, Inc., Burlingame, CA) or human plasma-absorbed rabbit anti-goat IgG (heavy and light chain specific) (Cappel Laboratories). The substrate for the peroxidase reaction was a solution of 0.04% ortho phenylenediamine dihydrochloride (Eastman Kodak Co., Rochester, NY) in citrate phosphate buffer with 0.012% hydrogen peroxide (Mallinckrodt, Inc., Paris, KY). Optical density was read at 30 min at 450 nm with a Titertek Multiscan (Flow Laboratories, Helsinki, Finland). Positive results were determined (a) by comparison of the absorbency of the test antiserum with that of an unrelated control antiserum and by comparison of the test antiserum on the test antigen and an unrelated control antigen and (b) by statistical comparison (paired t test).

MC5b-9 complex isolation. MC5b-9 complexes were isolated from sheep erythrocytes as described by Ware et al. (15). Briefly, antibody-sensitized sheep erythrocytes (EA) were incubated with human serum diluted in VBS⁺⁺ for 60 min at 37°C . The complement-coated EA membranes (EAC) were washed three times in BEP buffer, centrifuged at 27,000 g and 4°C for 20 min, solubilized with Triton X-100 (Sigma Chemical Co.) or Zwittergent ionic detergent 3-12 (SB12, Calbiochem-Behring, La Jolla, CA) for 30 min at room temperature. The detergent-to-protein weight ratio was 40:1. The mixture was centrifuged at 27,000 g for 20 min and the supernatant was concentrated on a YM-30 membrane (Amicon Co., Lexington, MA), and stored at 4°C . Purified MC5b-9 was obtained from EAC membranes solubilized in SB12 and isolated by Bio-Rad A 15 M chromatography (Bio-Rad Laboratories, Richmond, CA) as previously reported (15). Additional purified MC5b-9 was kindly provided by Dr. William Kolb (San Antonio, TX).

Reconstitution experiments were performed with EAC1-7 cells prepared as follows: $1.0\ \text{ml}$ of C8-deficient serum was incubated with 1.6×10^6 EA for 60 min at 37°C and then washed six times with cold VBS⁺⁺ to remove residual serum components. The absence of C9 on the EAC1-7 intermediates was demonstrated by the lack of aggregation of these cells by anti-human C9 antiserum. EAC1-7 intermediates were then incubated at 37°C for 60 min with 2,000 hemolytic units of C8 alone or 2,000 hemolytic units of both C8 and C9, or 2,000 hemolytic units of C9 alone. The EAC membranes were washed, solubilized, and concentrated by ultrafiltration as described above. Unlysed EA cells (those incubated with heat-inactivated serum or C8-deficient serum) were lysed during the BEP buffer washes. A similar experiment was performed in which solubilized EAC membranes were obtained from EA incubated in C9-deficient serum alone, or C9-deficient serum reconstituted with 2,000 hemolytic units of C9.

Serum C5b-9 (SC5b-9) was made by incubating NHS with LPS ($300\ \mu\text{g}/\text{ml}$ of serum) or cobra venom factor ($20\ \text{U}/\text{ml}$ of serum) for 1 h at 37°C . These sera were placed directly on microtiter plates for ELISA. Binding to the plates was similar whether antigens were incubated in coating buffer or in PBS.

Analysis of reactivity of Poly C9-MA with poly C9 and with monomeric C9. The interaction of Poly C9-MA with poly C9 and with monomeric C9 was investigated by ELISA and by a radioimmunoassay (RIA). For the RIA, C9 was labeled with ^{125}I by a lactoperoxidase procedure (34) at 4°C for 4 h. The specific activity of C9 was $110,000\ \text{cpm}/\mu\text{g}$ protein. Radiolabeled poly C9 was prepared from mono-

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meric ^{125}I -C9, with a specific activity of 10,400 cpm/ μg protein. The specific activity of monomeric C9 was made 10-fold greater than that of poly C9 to maximize the sensitivity of the reaction of Poly C9-MA with monomeric C9. To obtain monomeric C9 free of possible aggregates that might have formed during its purification and storage, 0.22 mg of ^{125}I -C9 was mixed with 2.4 mg of bovine serum albumin (BSA, Sigma Chemical Co.), applied to a $1.5 \times 100\text{-cm}$ Sephadex G-100 column (Pharmacia Fine Chemicals, Uppsala, Sweden) and eluted with PBS. Fractions containing the monomeric component were used directly in the immunoassays. The presence of functionally active C9 in these fractions was assessed by demonstrating the ability of this material to lyse EAC1-7 in the presence of limiting amounts of C8.

200 μl of immunobeads bearing rabbit anti-mouse Ig (Bio-Rad Laboratories), were incubated with 5 μl of Poly C9-MA or the control monoclonal MBM10 for 1 h at room temperature. The beads were washed three times with PBS containing 0.05% Tween and once with PBS at 4°C . The beads were then transferred to polypropylene tubes (Walter Sargent, Inc., Princeton, NJ) precoated with 1% BSA to prevent nonspecific adherence of ^{125}I -labeled material to the walls of the tube. To saturate the beads fully, 1 μg of radiolabeled monomeric C9 or poly C9 was incubated at room temperature for 1 h with the immunobeads, which were then washed as above, and the radioactivity was measured in a gamma counter.

To prepare a uniform ^{125}I -poly C9 standard, ^{125}I -poly C9 was incubated and stored in PBS-1% sodium-desoxycholate (DOC, Fischer Scientific Co., Fairlawn, NJ), thereby decreasing the aggregation of poly C9. Incubation in DOC did not change the dose-response binding characteristics of ^{125}I -poly C9 to Poly C9-MA. The DOC-treated material was applied to a Bio-Rad A15 M ($0.9 \times 42\text{ cm}$) column and two major peaks eluted with PBS at a flow rate of 5 ml/h. Fractions from the first peak that demonstrated positive binding to Poly C9-MA by RIA were pooled and stored at 4°C in PBS-1% DOC.

Dose-response curves of ^{125}I -poly C9 or ^{125}I -C9 were determined by adding increasing amounts of either material (protein concentrations of 1 $\mu\text{g}/\text{ml}$) to 50 μl of immunobeads that were incubated with 10 μl of a dilution of Poly C9-MA supernatant. The total amount of labeled material added was measured, the beads washed, and the amount of bound radioactivity counted.

Inhibition of the ^{125}I -poly C9 RIA by poly C9 and purified MC5b-9. Inhibition of ^{125}I -poly C9 binding to the immunobeads was accomplished using unlabeled cold poly C9 or purified MC5b-9. Immunobeads were incubated with Poly C9-MA supernatant, washed as above, and transferred in PBS-1% DOC to BSA-coated polypropylene tubes. Unlabeled poly C9 (protein concentration of 100 $\mu\text{g}/\text{ml}$) that had been stored in PBS-1% DOC was then added to the immunobeads in 10- μl increments. After 1 h of incubation on a rotating platform at room temperature, a constant amount of ^{125}I -poly C9 was added and total radioactivity measured. After 10 min of incubation at room temperature, the beads were washed and the bound counts determined. In other experiments, 10- μl increments of purified MC5b-9 (protein concentration of 100 $\mu\text{g}/\text{ml}$) were added under exactly the same conditions instead of unlabeled poly C9.

Patients. Kidney tissue was selected from two groups of patients. One group included 16 patients with glomerulonephritis: membranoproliferative glomerulonephritis (MPGN) type I ($n = 3$), MPGN type II ($n = 2$), membranous nephropathy ($n = 3$), SLE, $n = 3$, IgA nephropathy ($n = 3$), and anaphylactoid purpura nephritis ($n = 2$). Tissue

was obtained by percutaneous renal biopsy from 14 of these patients with serum creatinine levels ranging from 0.7 to 2.3 mg/dl. In two other patients, both with MPGN type II, the kidney tissue was obtained at the time of nephrectomy, before renal transplantation. The second group of patients included 17 patients with diseases other than glomerulonephritis: diabetes mellitus ($n = 7$), hypertension ($n = 3$), obstructive uropathy ($n = 3$), congenital dysplasia ($n = 2$), and amyloidosis ($n = 2$). Kidney tissue was obtained by percutaneous renal biopsy in eight patients (four with diabetes mellitus, and one each with hypertension, obstructive uropathy, congenital dysplasia, and amyloidosis); the serum creatinine of these patients ranged between 0.6 and 2.1 mg/dl. Kidney tissue from the other nine patients in the nonnephritic group was obtained at the time of nephrectomy, before renal transplantation. All tissues were evaluated and characterized by light and electron microscopy as previously described (35-37). Routine immunofluorescent studies were carried out with polyclonal antisera to human IgG, IgM, IgA, C1q, C3, C4, properdin, albumin, and fibrinogen (36, 38). The immunohistochemical localization of immunoglobulins and other antigens will not be reported in this paper. There was a good correlation between the deposition of immunoglobulin and C3 in kidney tissue from patients with glomerulonephritis, except for those with MPGN. In the latter disease, the deposition of C3 was more extensive than that of immunoglobulin (37). In tissue from patients with nonnephritic disease, a similar discrepancy was obtained between the deposition of C3 and that of immunoglobulin, as previously reported (36, 38).

RESULTS

Initial studies with Poly C9-MA. The monoclonal antibody (Poly C9-MA) [IgG₁, α] was developed after immunization with collagenase-digested normal human basement membranes. However, Poly C9-MA did not react on ELISA with either the collagenase-digested CBM material used for immunization or the known constituents of renal extracellular matrices: collagen type I, III, IV, V, fibronectin, and laminin. Each antigen was tested with an appropriate positive control antiserum. Immunohistochemical studies on human kidney specimens from patients with glomerulonephritis suggested a fluorescence pattern of Poly C9-MA similar to that observed for anti-C3 antisera. However, extensive absorption of Poly C9-MA with normal human plasma failed to diminish immunofluorescence. By ELISA, the optical density of the reaction of Poly C9-MA with 10 different samples of untreated normal human serum was 0.053 ± 0.02 (mean \pm SD) (Fig. 2). When each of these serum samples was activated with LPS, an OD of 0.193 ± 0.11 was seen ($P < 0.005$). This difference was significantly inhibited by heat inactivation of the serum before addition of LPS (0.076 ± 0.05 , $P < 0.01$). A similar positive interaction was seen when serum was activated with cobra venom factor; no binding was observed with cryoprecipitate as well as with serum treated with 0.02 M EDTA before LPS activation.

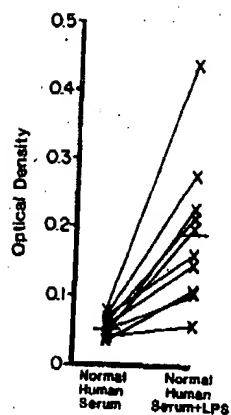


FIGURE 2 Comparison by ELISA of the interaction of Poly C9-MA with 10 different samples of normal human serum (OD [mean \pm SD], 0.053 \pm 0.02) and with the same samples of human sera activated with LPS (OD [mean \pm SD] 0.193 \pm 0.11) ($P < 0.005$).

Immunofluorescent reactivity of Poly C9-MA with complement-treated zymosan particles. Because Poly C9-MA appeared to bind to an antigen expressed only after complement activation, a more specific reaction with complement components was investigated on complement-treated zymosan particles. As shown in Table I, Poly C9-MA recognized an antigen present on zymosan incubated in NHS. This antigen was not detected after heat inactivation or divalent cation che-

lation of serum. However, Poly C9-MA reacted with zymosan particles incubated with sera deficient in C1r and C2, but not with sera deficient in any of the individual components C3 to C9. Reconstitution of C8 or C9-deficient sera with the functionally purified missing component restored Poly C9-MA fluorescence (Fig. 3, Table I). These data indicate that Poly C9-MA recognized an antigen formed by the reaction of C9 with C5b-8.

Interaction of Poly C9-MA with MC5b-9, poly C9 and other complement components analyzed with ELISA. Poly C9-MA reacted positively by ELISA with either 1 μ g of poly C9 or purified MC5b-9; this latter complex was obtained from EAC membranes by solubilization with SB12 and gel filtration chromatography (Table II). There was positive reactivity of polyclonal anti-human C9 with both poly C9 and MC5b-9 complexes, whereas the unrelated IgG1 monoclonal antibody MBM10 had no reactivity (Table II). When Poly C9-MA was reacted with 10-fold serial dilutions of MC5b-9 or poly C9 (initial protein concentrations of 100 μ g/ml), there was a similar linear decrease in reactivity (Fig. 4). The slopes of the MC5b-9 and poly C9 dose-dependent relationships as determined by linear regression analysis were -0.12 and -0.11, respectively. There was no binding of Poly C9-MA to purified C5, C8, C9, and to the intermediates C5b6 and C5b67 (Table II). Because of the nature of our ELISA, inhibition studies could only be carried out with low

TABLE I
Characterization of the Reactivity of Poly C9-MA with Zymosan Treated with NHS or Complement-deficient Sera

Sera used for zymosan incubation	Indirect immunofluorescent reactivity of serum-treated zymosan particles with complement antisera						Poly C9-MA*
	Anti-C3	Anti-C5	Anti-C8	Anti-C7	Anti-C8	Anti-C9	
NHS	+	+	+	+	+	+	+
Heat-inactivated or NHS + 10 mM EDTA	-	-	-	-	-	-	-
Complement-deficient sera							
C1r	+	+	+	+	+	+	+
C2	+	+	+	+	+	+	+
C3	-	-	-	-	-	-	-
C5	+	-	-	-	-	-	-
C6	+	+	-	-	-	-	-
C7	+	+	-	-	-	-	-
C8 α - γ chain	+	+	+	+	-	-	-
C8 β chain	+	+	+	+	-	-	-
C9	+	+	+	+	-	-	-
C8-deficient sera + C8							
C9-deficient sera + C9							

* As a control, no reactivity was demonstrated using an unrelated IgG₁ monoclonal antibody (MBM10) with specificity for human basement membrane.



FIGURE 3 Immunofluorescence of opsonized zymosan particles stained with Poly C9-MA (A). When zymosan particles were incubated in C9-deficient serum reconstituted with functionally purified C9, positive immunofluorescence was observed. $\times 480$. (B) Zymosan particles incubated in C9-deficient serum alone failed to react with Poly C9-MA. $\times 480$.

concentrations of Poly C9-MA. Therefore a 1:400 dilution of Poly C9-MA supernatant yielding an OD of 0.10 was selected. 1 μ g of purified MC5b-9 was incubated with Poly C9-MA for 1 h at room temperature and overnight at 4°C. The mixture was then centrifuged at 27,750 g for 30 min. The OD of the absorbed

Poly C9-MA was 0.03, which was identical to that of the control using the peroxidase-labeled antibody without the primary antibody.

To verify the requirement of C9 for recognition of Poly C9-MA, EAC1-7 and EAC1-8 intermediates were formed by incubating EA in complement-defi-

TABLE II
Binding of Poly C9-MA to Poly C9 and MC5b-9 but Not to Other Complement Component Intermediates

	Reactivity of antibodies with various substrates by ELISA				
	Poly C9-MA	Anti-C5	Anti-C8	Anti-C9	MBM10*
OD units					
Purified antigens					
Poly C9†	0.42			1.42	0.09
C5b-9§	0.62			1.29	0.09
C5b6	0.05	1.59			0.09
C5b67	0.06	1.59			0.09
C5	0.09	1.69			0.14
C8	0.14		1.56		
C9	0.09			1.70	0.12
Solubilized membranes					
MC5b-9	0.44			1.42	0.05
MC5b-8	0.11				0.08
MC5b-7	0.08				0.06
MC5b-8 + C9	0.23				0.08
NHS	0.07				0.07
Type I collagen	0.07	0.16	0.11	0.26	0.08

* Monoclonal antibody (IgG₁) reactive with basement membranes.

† 1 μ g of poly C9 was used for each test.

§ 1 μ g of purified MC5b-9 was used for each test.

^{||} Solubilized membranes obtained from EA incubated in heat inactivated serum reacted with Poly C9-MA yielded an OD of 0.02 (MBM10, 0.04).

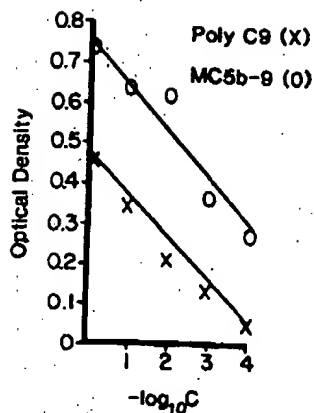


FIGURE 4 Linear regression analysis of Poly C9-MA binding on ELISA to serial 10-fold dilutions of Poly C9 and MC5b-9 demonstrated similar linear relationships. The initial protein concentrations (C) of poly C9 and MC5b-9 were 100 $\mu\text{g}/\text{ml}$. The slope of the Poly C9 relationship was -0.11 , while that of MC5b-9 was -0.12 ($r = 0.95$ for both reactions).

cient sera. Because of limited amounts of complement-deficient sera, purification of these complexes by gel filtration chromatography was not feasible. Membranes solubilized by Triton X-100 from EA cells incubated in NHS reacted with Poly C9-MA in a manner similar to that of purified MC5b-9 (Table II). As a control, solubilized membranes that had been incubated in heat-inactivated serum did not bind to Poly C9-MA. EAC membranes bearing C1-7 were produced by incubating EA in C8 deficient serum. EAC1-8 was formed by the addition of functionally purified C8 to the extensively washed EAC1-7, resulting in hemolysis. A control was performed by adding C9 alone to the EAC1-7 cells without the addition of C8. EAC membranes were then solubilized with Triton X-100 and reacted on ELISA with Poly C9-MA and the control monoclonal antibody MBM10. As shown in Table II, Poly C9-MA reacted with EAC membranes containing MC5b-9 obtained after incubation with fully reconstituted sera and not with EAC1-7 or EAC1-8 solubilized membranes. Similar results were obtained when EA were incubated in C9-deficient serum or C9-deficient serum reconstituted with functionally pure C9.

Interaction of Poly C9-MA with poly C9, monomeric C9, and MC5b-9 determined by RIA. In a solid-phase RIA system, Poly C9-MA was incubated with ^{125}I -poly C9 or ^{125}I -monomeric C9. In the first experiment, excess poly C9 was used to fully saturate the binding capacity of the Poly C9-MA attached to the immunobead. Poly C9-MA demonstrated a specific uptake of $0.174 \mu\text{g}$ of ^{125}I -poly C9. MBM10 served as a negative control and gave an uptake of $0.008 \mu\text{g}$ of ^{125}I -poly C9. Under the same conditions, the uptake

of monomeric C9 by Poly C9-MA was $0.002 \mu\text{g}$ of ^{125}I -C9, which is only 1.4% of the uptake of poly C9. When ^{125}I -poly C9 was added in increasing amounts, a dose-response curve was obtained (Fig. 5 A). Increasing the amounts of ^{125}I -C9 did not induce binding to Poly C9-MA.

When increasing amounts of either unlabeled poly C9 or purified MC5b-9 were added to a constant amount of ^{125}I -poly C9, identical dose-response inhibition curves were obtained (Fig. 5 B). Although there was no inhibition of this RIA with $50 \mu\text{l}$ of NHS (bound/free ratio of 1.7), there was inhibition by the addition of $50 \mu\text{l}$ of human serum activated with LPS (a bound/free ratio of 0.40).

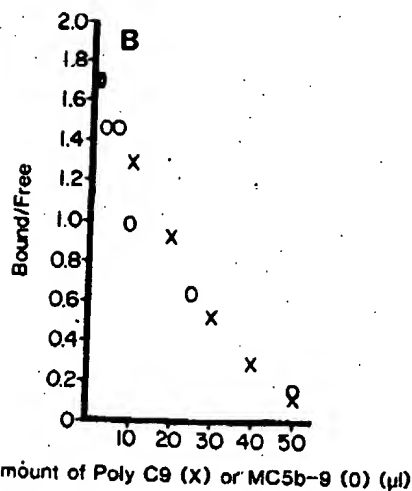
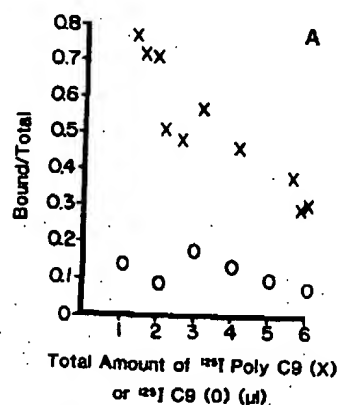


FIGURE 5 (A) RIA of Poly C9-MA: A dose-response relationship was observed for ^{125}I -poly C9 but not for increasing amounts of ^{125}I -monomeric C9. In this assay, the concentrations of C9 and poly C9 were $1.0 \mu\text{g}/\text{ml}$. (B) Increasing amounts of either unlabeled MC5b-9 or poly C9 (protein concentrations of $100 \mu\text{g}/\text{ml}$) inhibited the binding of a constant amount of ^{125}I -poly C9 in an identical manner. Addition of normal human serum ($50 \mu\text{l}$) did not inhibit binding (bound/free = 1.7).

Immunofluorescence studies. Poly C9-MA binding was initially recognized on the internal elastic lamina and media of vessels and focally in the juxtaglomerular zone and mesangial stalk region of normal adult kidney sections (Fig. 6). There was no detectable immunofluorescence with normal fetal kidney; with other regions of mature human kidney, muscle, or skin; or with isolated lymphocytes, monocytes, polymorphonuclear leukocytes, or platelets. However, immunofluorescent positive vessels were observed in normal human liver and spleen. By phase-epifluorescence microscopy, Poly C9-MA was often recognized on phase-dense material or particles in extracellular membranes. These densities did not stain with hematoxylin-eosin, Masson Trichrome, periodic acid-Schiff, Von Kossa, Prussian blue, sudan black D, or colloidal iron stains.

The distribution and intensity of Poly C9-MA and of polyclonal antisera to human C3, C5, C6, C7, C8, and C9 were assessed on kidney sections from a variety of renal diseases. In general, Poly C9-MA bound to sites that reacted with goat antisera to the individual human complement components C5, C6, C7, C8, and C9. However, the intensity of staining was different. There was uniform conjunction of Poly C9-MA and anti-C5, which stained all tissue strongly. Antisera to C6 and C7 reacted with trace intensity, whereas anti-C8 and anti-C9 were recognized with moderate intensity. When visible, these antisera localized in the same areas as Poly C9-MA staining.

When the distribution of the MAC (as interpreted by reactivity with Poly C9-MA) was compared with that of C3, the pattern found in tissue from patients with glomerulonephritis was different from that in tissue from patients with nonnephritic diseases. In MPCN (type I and II), IgA nephropathy and anaphylactoid purpura, SLE, and membranous nephropathy, there

were areas of homology in the distribution of C3 and the MAC (Fig. 7). In MPCN type II, Poly C9-MA and anti-C3 encircled dense deposits in the glomerular and tubular basement membranes, and both antisera demonstrated mesangial rings typical of dense deposit material (37). In IgA nephropathy and anaphylactoid purpura, the mesangial regions contained both the MAC and C3, although the latter was also trapped along the internal aspect of the GBM. In SLE, there was a close correlation between the distribution of C3 and the MAC in the glomerular mesangium and in subendothelial GBM deposits. In membranous nephropathy, C3 and the MAC were present in epimembranous deposits (Fig. 8). However, in addition to areas of homology in these diseases, the MAC was frequently present in a coarse granular pattern (in phase dense areas) along tubular basement membranes in loci where C3 was consistently absent.

In diabetes, hypertension, obstructive uropathy, amyloid nephropathy, and congenital dysplasia, the MAC was present in greater abundance and frequently in different loci than C3. The MAC appeared in a coarse granular pattern along tubular basement membranes and Bowman's capsule in close conjunction with the phase-dense granules previously mentioned (Fig. 9). The MAC deposition in vessel walls, internal elastic lamina, juxtaglomerular and mesangial regions and in areas of sclerosis was characteristically extensive (Fig. 10). The MAC was generally not observed trapped along the internal aspect of the GBM, except in a focal granular pattern (Fig. 11). In contrast, C3 was present focally in a linear pattern along basement membranes of the glomeruli, tubules, and Bowman's capsule; in a granular pattern in peripheral and stalk regions of the mesangium; and also in arteriolar media and intima, and frequently in the lumina of vessels. There

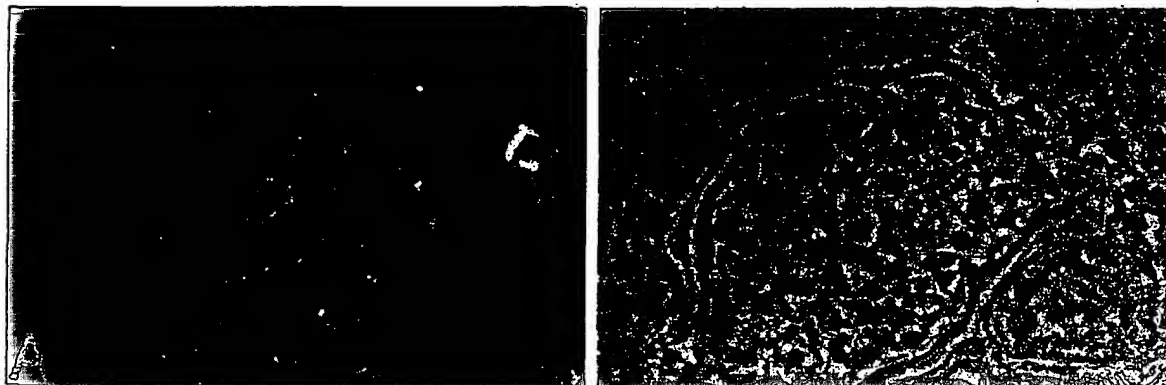


FIGURE 6 Immunofluorescence of a normal human glomerulus stained with Poly C9-MA (A) Poly C9-MA reacted with the internal elastic lamina and media of a small vessel (arrow), and weakly in a coarse granular pattern in the glomerular mesangial region. $\times 180$. (B) A phase-contrast microscopic picture of the same glomerulus. Note small arteriole (arrow). $\times 180$.

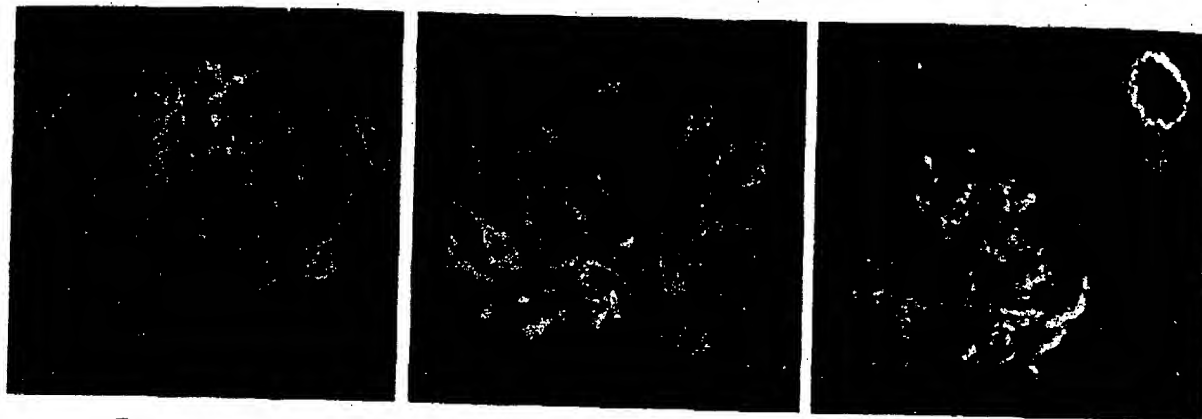


FIGURE 7 Immunofluorescence of Poly C9-MA on glomeruli from patients with (A) MPCN (type II) demonstrating fluorescence of the GBM and the mesangium, which on higher power reveal mesangial rings; (B) SLE with mesangial deposits; (C) IgA nephropathy with mesangial fluorescence. Staining of arteriole (arrow) is similar to that seen in normal human kidney tissue. A, B, C, $\times 200$.

was good correlation between the areas of deposition of the MAC and (a) periodic acid-Schiff-positive material seen in the diabetic mesangium, (b) C3 deposition in diabetic fibrin caps and capsular drops, (c) areas of thioflavin T staining in amyloidosis, (d) hyalinization of hypertensive glomeruli and vessel walls, and (e) sclerosis in all forms of endstage renal disease.

When Poly C9-MA was absorbed with purified poly C9, there was complete abrogation of immunofluorescent staining on normal adult and diseased diabetic kidney tissue. Absorption with normal human plasma, diabetic plasma, and cryoprecipitate had no effect on Poly C9-MA immunofluorescence.

DISCUSSION

Complement-induced membrane damage is effected by the MAC, a multimolecular complex composed of the five terminal complement components. After cleavage of C5 into C5a and C5b, C6 and C7 attach to C5b, forming the C5b67 complex, which interacts with hydrophobic regions of lipid bilayers. The addition of C8 and C9 enhances the interaction of the complex with the membrane interior (5, 6, 39). In the process of MAC assembly, the terminal components undergo conformational changes, creating binding sites for phospholipids that are released from and

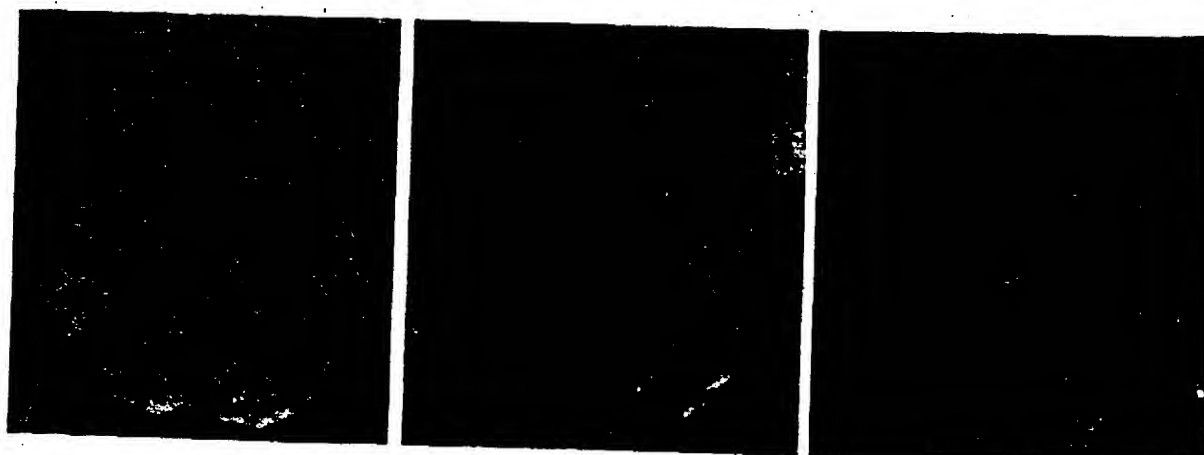


FIGURE 8 Immunofluorescence of a glomerulus in a patient with membranous glomerulopathy. (A) Poly C9-MA stained in a granular pattern along the GBM. $\times 220$. Anti-C5 antibody stained with a similar intensity and distribution. (B) Staining with polyclonal anti-human C3 antibody $\times 220$. (C) There was minimal staining with polyclonal anti-C9; reactivity with anti-C8 was equivalent in intensity and distribution to anti-C9. Anti-C6 and anti-C7 reacted with only trace intensity. $\times 220$.

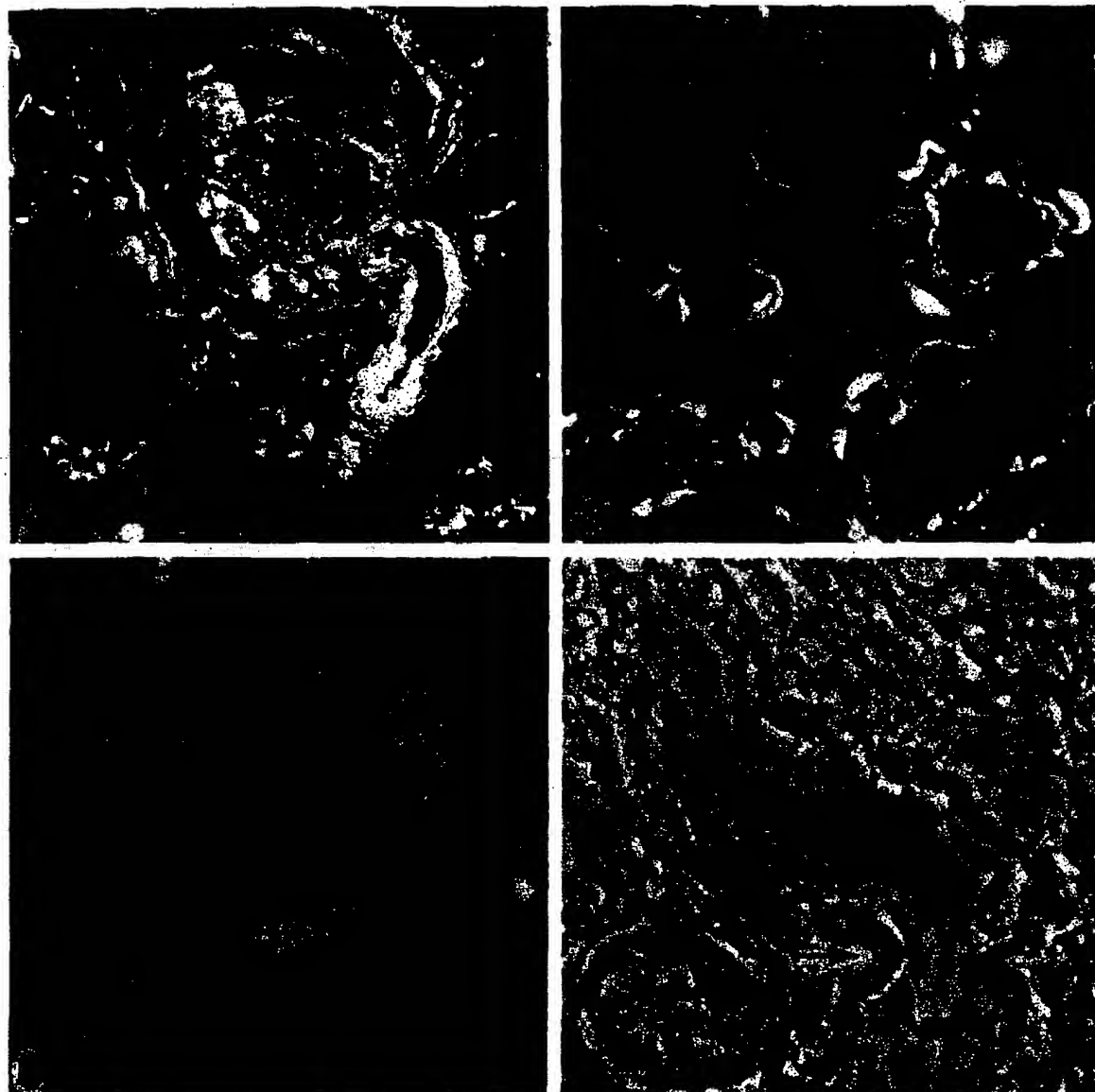


FIGURE 9 Immunofluorescence of Poly C9-MA in diabetic nephropathy (A) Extensive granular staining of Poly C9-MA in the mesangium, Bowman's capsule, afferent and efferent arterioles, and tubular basement membranes. $\times 280$. (B) While Poly C9-MA staining was present on the inner and outer laminations of diabetic tubular basement membranes, polyclonal antibody to human C3 was absent from the inner ring (see arrow), where Poly C9-MA was present in a coarse granular pattern. $\times 630$. (C) Localized immunofluorescence of Poly C9-MA on diabetic tubular basement membrane (arrows) corresponding to phase-dense material seen by phase-contrast microscopy of the same section (D). C and D, $\times 775$.

which cause the reorganization of membrane lipid bilayers (39). The end result of MC5b-9 attachment is membrane damage, although the precise mechanism whereby the MC5b-9 complex disrupts the membrane barrier is uncertain. A transmembrane lipid channel may be formed because of lipid bilayer reorganization (4, 6), or, alternatively, the proteins of the MC5b-9

complex (40, 41) or poly C9 (8) alone may form a transmembrane channel.

Prior studies have shown that the assembly of the C5b-9 complex leads to the appearance of neoantigens associated with C5b6, C5b67 (7), and poly C9 (9).

This study has characterized a monoclonal antibody, Poly C9-MA, which recognizes a neoantigen of the C9



FIGURE 10 Immunofluorescent staining with Poly C9-MA was observed in vessels of kidney tissue obtained from a variety of nonnephritic patients. Tissue from a patient with hypertensive nephrosclerosis demonstrating intense immunofluorescence of the glomerulus and afferent-efferent arterioles (A, $\times 200$) and the media and internal elastic lamina of a larger artery (B, $\times 410$). (C) An artery from a patient with amyloidosis; while there was little homology with antisera to C3 in this vessel, there was a close correlation with thioflavin T staining. $\times 200$.

portion of the C5b-9 complex. Evidence that the antigen reacting with Poly C9-MA is not detected in NHS but requires complement activation is derived from a number of observations. First, it was not possible to inhibit immunofluorescent reactivity of Poly C9-MA on normal human and diseased kidney sections by absorbing the antibody with normal human serum or plasma. Second, there was a lack of binding of Poly C9-MA to NHS on ELISA, but positive reactivity with complement-activated serum. Third, Poly C9-MA immunofluorescence of zymosan particles preincubated with NHS was abrogated by heat inactivation or divalent cation chelation of serum. Fourth, Poly C9-MA failed to bind to purified complement components on ELISA, and to monomeric native C9 in a sensitive RIA.

Finally, human serum inhibited the ^{125}I -poly C9 RIA only after complement was activated by LPS.

That Poly C9-MA recognizes a neoantigen of C9 and not other portions of the MAC was demonstrated in two different assay systems. The zymosan immunofluorescence assay demonstrates the absence of Poly C9-MA binding to zymosan particles incubated in sera deficient in individual terminal components, and the restoration of fluorescence by reconstitution of C9-deficient sera. These results were corroborated by Poly C9-MA binding on ELISA to EAC membranes that included C5b-9, but not to intermediate complement complexes. The binding to purified polymerized C9 demonstrated by ELISA and in the RIA without reactivity to monomeric C9 documents the specificity of

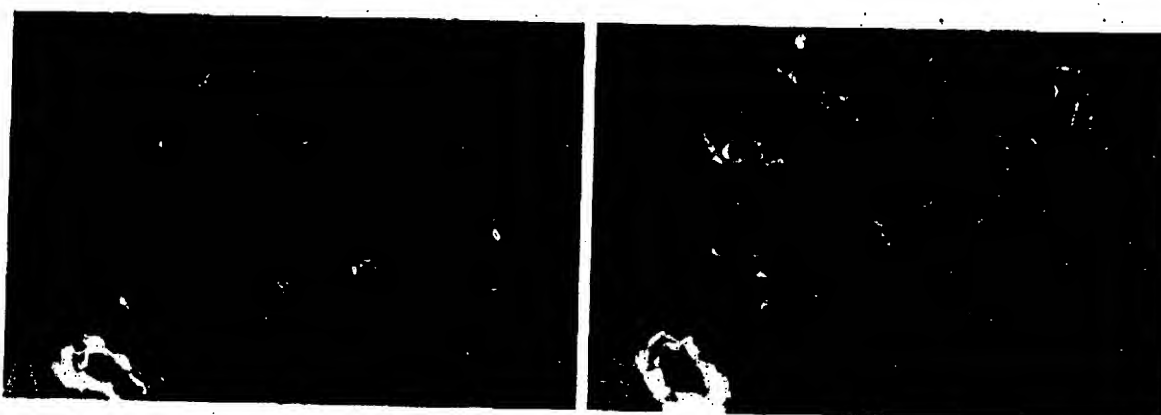


FIGURE 11 Immunofluorescence of a normal human glomerulus demonstrating the contrast between (A) Poly C9-MA staining of an afferent arteriole and focally within the mesangium and (B) anti-C3 antisera staining of the same afferent arteriole and mesangium but also present along the inner aspect of the GBM. Polyclonal anti-C9 antisera stains this glomerulus in a pattern identical to that of Poly C9-MA.

Poly C9-MA. There is compelling evidence that the antigenic determinant recognized by Poly C9-MA is common to both MC5b-9 and poly C9: There was identical dose-response inhibition of the ¹²⁵I-poly C9 RIA by unlabeled poly C9 and by purified MC5b-9, the slopes of the MC5b-9 and poly C9 dose-response relationship on ELISA were similar, there was inhibition on ELISA of poly C9 reactivity by MC5b-9 absorption of the antibody, and there was elimination of Poly C9-MA staining on normal and diseased human kidney by absorption with poly C9.

The development of a monoclonal antibody to the MAC from collagenase digested GBM may appear fortuitous. However, the GBM preparation is only partially purified and is known to consist of a variety of renal extracellular matrix and plasma antigenic determinants (42, 43). That Poly C9-MA did not react with the preparation used for immunization reflects the scarcity of the antigen in this preparation, as well as the capacity to develop monoclonal antibodies to trace amounts of an antigen. Although the possibility that Poly C9-MA reacts with an epitope on an unrelated extracellular matrix protein is difficult to exclude, this likelihood is diminished by the lack of reaction on ELISA to known collagens or constituents of renal extracellular matrices (collagen types I, III, IV, V, fibronectin, and laminin), and the negative immunofluorescence of normal fetal kidney, human muscle and skin, and extracellular basement membranes of the adult human kidney.

Recently, Biesecker et al. (13) have demonstrated by immunofluorescence that a polyclonal antiserum to MAC binds to deposits in the kidney of patients with SLE nephritis. Although this antiserum recognizes a neoantigen(s), its specificity has not been characterized. A similar distribution of IgG, C1q, C4, and MAC was noted in glomeruli and blood vessels, whereas MAC was more frequently present in tubular deposits. Similar results were observed for the distribution of Poly C9-MA in various forms of glomerulonephritis. There was a close correlation in these diseases between the deposition C3 and that of the MAC in glomerular and mesangial regions. However, Poly C9-MA stained loci in tubular basement membranes that did not react with anti-C3, a finding which corroborates the observation of Biesecker et al. (13) in SLE.

In nonnephritic renal diseases, the MAC was present extensively in the mesangial and juxtaglomerular regions of glomeruli, internal elastic lamina and media of blood vessels, and tubular basement membranes. The deposition of the MAC was especially evident in tissues with sclerosis. In all of these loci, particularly in tubular basement membranes, there was little correlation between the distribution of C3 and that of the MAC. Additionally, the MAC was not present in loci

such as the lumen of vessels and capillaries or along the internal aspect of the GBM where plasma proteins like C3 and albumin have been found. The presence of complement components in renal tissue from diseases such as hypertension, diabetes mellitus, obstruction, congenital dysplasia, and amyloid has been documented. In an immunohistochemical study of end-stage kidney disease, it has been noted previously that immunoglobulins and classical and alternative pathway complement components were present in hyalinizing glomeruli, regardless of the cause of renal failure (36). Verroust et al. (44) found that multiple complement components were detected by immunofluorescence in immunoglobulin-mediated glomerular injury. Even in instances where immunoglobulin was absent, alternative pathway complement components were present. The presence of the MAC in diseased tissue is more indicative of complement activation than demonstration of individual complement components, which may reflect nonspecific trapping, as previously suggested. The deposition of the MAC in small arterioles of normal adult kidney tissue and its absence in fetal specimens is difficult to explain, but may indicate low-grade complement activation in these sites.

Whether the membrane attack complex is damaging renal extracellular matrices in a manner analogous to cell membrane disruption is uncertain, but the extensive presence of the MAC in all diseased renal tissue, especially in areas of sclerosis, suggests that complement activation may play a role in sclerosis of end-stage renal disease.

The frequent disparity in the distribution between the MAC and C3 in nonnephritic diseases was consistently observed. The reason for this difference is obscure, but may be explained in a number of ways. First, C3 may be present in a form not identified by polyclonal anti-C3 antiserum. Secondly, the initiation of attack complex activation may occur at a site distant from the MAC deposition, analogous to the observations of Gotze and Müller-Eberhard (45) who have shown that the C423 enzyme can affect the binding of C5, C6, and C7 to cell surfaces physically separated from where the C423 enzyme was bound. Thirdly, Polley and Nachman (46) have described C5b-9 activation by an interaction of platelet membranes, thrombin, and C3b that appears to be independent of the classical or alternative activation pathways. Although C3b is necessary for platelet-induced C5b-9 formation, its uptake on platelet membranes was less than that observed for C8. A similar phenomenon may occur on diseased renal basement membranes. Finally, C5b-9 formation or C9 polymerization may be induced by a mechanism not previously described and not requiring binding of C3. In this regard, it is of interest that a disparity also exists between the exten-

sive deposition of the MAC and the limited localization of immunoglobulin, especially in tissue from patients with renal disease other than glomerulonephritis.

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